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Thank you.

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Dendritic Cells as Antigen Presenting Cells *in Vivo*

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The biology of antigen presenting cells (APC) traditionally is studied in tissue culture systems using T cells that have been expanded beforehand by stimulation with antigen. Here we consider the distinctive roles of dendritic cells for sensitizing or priming T cells both *in vitro* and *in vivo*. Several functions of dendritic cells have been identified in tissue culture that are pertinent to T cell sensitization. These include the ability to a) capture and retain foreign antigens in an immunogenic form, b) bind antigen-specific resting lymphocytes, and c) activate T cells to produce lymphokines and undergo long term clonal growth. Dendritic cells have several properties *in vivo* that also would contribute to APC function. These are a) their widespread tissue distribution permitting access to antigens in most organs, b) the capacity to home via the blood stream and afferent lymph to the T-dependent areas of spleen and lymph node, and c) the ability to capture antigen in antigen-pulsed animals. Dendritic cells bearing antigen have been administered *in situ* to initiate responses like contact sensitivity, graft rejection, and antibody formation. A most striking recent example is that, when dendritic cells are pulsed with protein antigens *in vitro* and administered to immunologically naive mice, there is direct priming of antigen-specific T cells that are restricted to the MHC of the injected APC.

KEYWORDS: APC *in vivo*, Langerhans cells, MHC-restricted priming, mixed leukocyte reaction, antibody response

INTRODUCTION

The very first studies of dendritic cell function in tissue culture documented their capacity to initiate T-dependent immune responses [Table I]. This function was far more developed in dendritic cells than in other types of antigen-presenting cells or APC [1-8].

By "initiate T-dependent immune responses," we mean the capacity to induce or sensitize resting, particularly unprimed, T cells so that they begin to grow and make lymphokines and cytolytins. Sensitization is not simply a matter of antigen processing and presentation. The latter yield peptide-MHC complexes that are the ligand for the clonotypic T cell receptor. Previously activated T cells, particularly T-T hybrids, will recognize and produce IL-2 when challenged with peptide-MHC complexes on aldehyde-fixed APC or on lipid bilayers, implying that a ligand for the TCR is sufficient for triggering these T cells. Yet fixed dendritic cells do not stimulate primary immune responses from resting T cells [25]. What

TABLE I

Primary Responses Induced by Dendritic Cells *in Vitro*

The primary antibody response by mixtures of B and T lymphocytes to foreign red cells and hapten-carrier conjugates [5, 7, 9-11]
The primary mixed leukocyte reaction by T lymphocytes to MHC-mismatched stimulator cells [1, 2, 4, 6, 12-14]
The induction of cytolytic T cells to haptens [15], viruses [16, 17], and transplantation antigens [18-22]
The activation of T cells that are specific for foreign proteins in the context of self MHC molecules [23, 24]

live dendritic cells are specialized to do is to accomplish the task of antigen presentation and T cell sensitization such that antigen-specific T cells grow, produce lymphokines, and become capable of active binding to other types of APC [25].

IN VITRO PROPERTIES OF DENDRITIC CELLS THAT CONTRIBUTE TO APC FUNCTION

Antigen Presentation

Recent studies in mouse spleen [26] and epidermal [27, 28] suspensions reveal that foreign proteins are captured only for a short time in the life history of a dendritic cell. Capture of several proteins—sperm whale myoglobin, ovalbumin, human gamma globulin, conalbumin—proceeds efficiently shortly after the dendritic cells are isolated from spleen and epidermis. After a day in culture, however, the dendritic cells cannot be pulsed with a native protein antigen even though these APC still present peptide fragments and such other stimulants as allogeneic class I and II MHC molecules, lectins, periodate, and anti-CD3 mAb [27, 29, 30]. In the latter cases, the ligand for the T lymphocyte may be available at the cell surface without a need for the uptake and processing of protein in culture.

Once pulsed with antigen, dendritic cells exhibit two interesting features. First, very few dendritic cells are needed to stimulate antigen-primed T cells, typically 100–300 times less than a standard APC population like mouse spleen [27]. This could mean that the dendritic cell is the principal APC in a spleen, or that other APC in spleen present much less effectively than dendritic cells. A second feature is that one can wait at least 1–2 days after pulsing, and the dendritic cells are still active as APC [26]. This indicates that the peptide-MHC complexes are stable on the dendritic cell surface, or that such high levels of MHC-peptide complexes have accumulated that a loss of peptide is insufficient to fall below the level needed to stimulate the T cell. Dendritic cells do express high levels of class I and II MHC products [31–37], but at present, the only way to assess the level of peptide-MHC complexes is to assess the capacity to stimulate antigen-reactive T cells. Such an approach also reflects other “costimulatory” features which may be better developed in dendritic cells.

These antigen presenting features of dendritic cells make them ideal “sentinels” for the onset of an immune response. Dendritic cells only capture antigens for a short period, perhaps where antigens are first deposited *in situ* as in the skin (Langerhans cells) or in the marginal zone of spleen (where most spleen dendritic cells seem to be found; see article by

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Agger, Crowley, and Witmer-Pack in this volume). Then the antigen is retained in an immunologically active form for a relatively long period during which time the dendritic cell can move to the T areas of lymphoid tissues, and find the complementary or antigen-specific T cell as described below. By downregulating antigen processing, dendritic cells may not displace the acquired antigen with other antigens or with self proteins after leaving the site of antigen deposition. Instead the original antigen predominates and is used to alert the immune system that a foreign protein has entered the body.

The antigen presenting potential of dendritic cells seems to be discounted by many investigators, because these cells are quantitatively weak at endocytosis especially relative to macrophages. While it may be easy to visualize endocytic uptake in macrophages, much of the uptake that is observed seems destined for destruction rather than presentation [38]. Therefore the ease with which one visualizes endocytosis in different APC is not directly correlated with the APC's presenting function. Very small amounts of endocytosis are evident in dendritic cells and other nonphagocytes like the B cell, but this requires the use of an appropriately sensitive tracer like rhodamine-ovalbumin [26]. It is likely that these small pools of endocytosed material, while difficult to visualize, are the relevant ones for presentation.

Endocytic activity again is an issue in considering the presentation of more complex antigens, such as infectious agents and whole cells. There are results indicating that dendritic cells are potent APC for influenza [17], Sendai and Moloney leukemia virus [39], Herpes Simplex [16], and mycobacteria [40]. Yet dendritic cells are demonstrably weak in phagocytic activity. At some stage of their life history, dendritic cells conceivably phagocytose small but relevant amounts of particulate antigens. Alternatively it has been hypothesized that dendritic cells contain surface proteases that process extracellularly [40]. A related possibility is that dendritic cells temporarily enclose particulates within their actively motile cytoplasmic processes, and that brief periods of "reversible engulfment" suffice for the processing of particulate antigens.

Binding and Retention of Antigen-specific T Cells

Dendritic cells efficiently capture antigen-specific T cells in a primary culture. Binding can be measured as the formation of cell aggregates or clusters, which can be isolated and quantitated [25, 41, 42]. These contain one or many dendritic cells and dozens to hundreds of T lymphocytes. A dendritic cell on average can drive 10-20 T cells into cell cycle in a day [30]. Cluster formation is observed with both CD4⁺ [25, 41-45] and CD8⁺ [19, 21] antigen-specific T cells.

Dendritic cells are capable of active movement, constantly sending out and retracting dendrites or long sheets of cytoplasm [46, 47]. Dendritic cells also temporarily bind T cells in the apparent absence of antigen [24, 48], but binding at least initially does not seem to require any of the known leukocyte integrins including LFA-1/CD11a [42, 44]. The working hypothesis is that the motility of dendritic cells permits the surveillance of T cells by a currently unidentified but antigen-independent mechanism. When there is complementarity between antigens presented by the dendritic cell and the clonotypic receptor, the T cell is retained and stimulated.

Once bound, T cells are retained in contact with dendritic cells for long periods, at least 24h or more [25, 41]. Prolonged contact may be essential for T cell activation given the data

that mitogens must be available for 12h before T cells become responsive to exogenous growth factors [49].

The T blasts that have been generated by dendritic cells are in turn capable of interacting with other APC, such as macrophages and B cells in the case of CD4⁺ blasts [23, 25, 50, 51], and a variety of leukocytes and other targets in the case of CD8⁺ blasts [19, 21]. The T blast-APC interaction allows the effector limb of the immune response to proceed. In the examples just cited, macrophages are induced to make IL-1 and other cytokines, B cells are stimulated to grow and make antibody, and targets for CTL are lysed. The potency of the sensitized T blast is impressive, as illustrated in T-B cell interactions [23, 25]. If one tries to stimulate an antibody response in a dendritic cell-depleted mixture of B and T cells, even from carrier-primed mice, one observes little or no response with several million T cells in the culture. Dendritic cells must be added to initiate the response. However, if one primes unsensitized T cells with dendritic cells over a 4–5 day culture, a few thousand *in vitro* sensitized T blasts induce vigorous B cell growth and antibody production in the absence of additional dendritic cells [23, 25].

The formation of dendritic-T cell contacts may be the rate-limiting event at the onset of an immune response. There is as yet little evidence that lymphocyte activating factors like IL-1 or IL-6 play a role in a primary, antigen-specific, CD4⁺ T cell response. Dendritic cells are not known to make these candidate activating factors [52–57], and antibodies to IL-1 [51, 58] and to IL-6 (unpublished) do not block dendritic cell function. When interacting dendritic cells and T cells are placed in one chamber of a two chamber culture vessel, and antigen-bearing B cells and T cells are placed in the second chamber, IL-2 is released into the second chamber but no T cell response (blastogenesis, DNA synthesis) occurs [48]. These results indicate strongly that an initial contact event is critical for the response to be initiated. [48, 59].

T Cell Activation

Following contact with dendritic cells, T lymphocytes begin to enlarge [21, 25], express activation antigens like class II MHC molecules and the low affinity IL-2 receptor [21, 41], and secrete a large panel of lymphokines [25, 49, 60–62]. The latter, in primary cultures, include IL-2, IL-4, IFN- γ , a T cell replacing factor most likely IL-5, and an as yet unidentified B cell growth factor. Other APC like monocytes and B cells do not initiate the formation of lymphoblasts or these lymphokines. However if the B cell is activated with anti-Ig coupled to an insoluble matrix, then the B blast can bind and activate T cells in the mixed leukocyte reaction [42]. As mentioned above, these "activation" properties of dendritic cells apparently do not require the release of factors like IL-1 and IL-6. Cytokines like IL-1 and GM-CSF can appear to be T cell activating factors, because they can amplify the function of dendritic cells [63–65].

Prolonged clonal expansion of T cells may also be regulated by the dendritic cell [66]. APC are essential in a system wherein single T cells are cloned in the presence of lectin and IL-2. The dendritic cell is at least 100 times more active than blood monocytes in supporting long term expansion.

In summary, dendritic cells have been shown, in defined *in vitro* systems, to carry out three critical properties of an APC: to acquire and retain antigens in an immunogenic form, to identify and retain antigen-specific T cells for periods of greater than a day, and to activate these T cells to grow and make needed lymphokines and cytolytins.

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Widespread Tissue Distribution

As outlined elsewhere in this volume (see articles by Holt *et al.*, Hart and MacKenzie), dendritic cells are widely distributed in nonlymphoid tissues, either in the epithelium as in epidermis [29], or in interstitial zones of many organs like heart, kidney, gut and endocrine organs [35, 36, 67-74]. The studies of Fabre have emphasized that most organs have two populations of interstitial leukocytes in the steady state: macrophages and dendritic cells [75]. The dendritic cells seem to express, at least in the rat, larger amounts of MHC class II and the leukocyte common antigen. An exceptional tissue in this regard is the brain, since Ia⁺ dendritic cells have yet to be identified in the steady state [36], whereas resident macrophages (microglia) are abundant [76].

What is unclear is whether tissue dendritic cells constitutively are able to capture antigens and to stimulate T cells, or whether these events must be triggered, perhaps by the antigen itself or by cytokines released locally upon antigen deposition. It is evident that the capacity of dendritic cells to bind and activate T cells can be induced and maintained by GM-CSF [64, 65]. A more recent finding is that of Larsen *et al.*, who visualized dendritic cells in epidermal sheets following skin grafting [77]. Dendritic cells in the epidermis are known to turn over slowly, with a half time of a month or more [78]. Yet within hours after grafting, even if the skin was placed on a syngeneic recipient, the dendritic cells began to enlarge, express more Ia, and to move out of the epidermis and into the dermis. Similar events took place in organ cultures of skin. Therefore dendritic cells in the steady state seem to be activated following the "trauma" of transplantation, and this sort of activation could contribute to the onset of an immune response.

Homing to the T-dependent Areas of Lymphoid Tissues

Dendritic cells can gain access to the lymph and to the blood [3, 4, 33, 47, 79-83] and from there, home to the T-dependent region of lymph nodes and spleen respectively [84-86]. The capacity of dendritic cells to home to the T area places these APC in the path of the recirculating T cell pool, i.e., in a position that is ideal for identifying the low frequency of T cells that can recognize the antigen(s) being carried by the dendritic cells.

Antigen Capture *In Situ*

There are several instances in which antigens have been administered *in vivo*, and then the dendritic cells are shown to be carrying that antigen in an immunogenic form (Table II). The latter is assessed by coculturing "*in vivo* pulsed" dendritic cells with antigen primed T cells, and documenting IL-2 release or DNA synthesis that normally is only induced with *exogenous* antigen.

Crowley *et al.* have recently used a group of mAb to provide evidence that dendritic cells are a major reservoir for immunogen *in situ*, at least in mouse spleen [88]. Foreign proteins were administered via the i.v. or i.p. route, and then spleen cells were isolated and tested for their capacity to present antigens to protein-specific T cell clones, or to bulk primed lymph node T cells. APC function was evident, maximally at 2h but lasting about a day, and the dendritic cells seemed to be the principal cell involved. This was shown using the 33D1 mAb

TABLE II

Examples of Antigens Captured by Dendritic Cells *in Situ*

Sperm whale myoglobin and other proteins by mouse thymus [87] and spleen [88] dendritic cells.
 Contact-sensitizing agents by dendritic cells in draining lymph nodes [89, 90].
 Ovalbumin by dendritic cells in rat lung [35] and sheep afferent lymph.

[92] to kill dendritic cells selectively with rabbit complement, and with the N418 mAb [93] to selectively sort dendritic cells on the FACS. The findings illustrate nicely the difference, alluded to above, between the level of bulk endocytic activity and the level of immunogen for T cells. Macrophages are known to be the main site for antigen uptake and destruction following administration of an antigen *in vivo*, yet dendritic cells seem to be the major source of immunogen.

A long known and distinctive function of dendritic cells is their capacity to stimulate syngeneic T cells in the "syngeneic mixed leukocyte reaction" [18]. This response often is envisaged as a form of autoractivity. It now seems possible that syngeneic responses represent reactions by specific T cells to those antigens that are being carried by dendritic cells *in situ*. This hypothesis might explain the fact that it has been so difficult to maintain these "autoreactive" T cells as clones, because there is no source of exogenous antigen to sustain long term growth.

EVIDENCE THAT DENDRITIC CELLS INDUCE IMMUNE RESPONSES *IN SITU*

The induction of immune responses *in situ* typically is performed with antigens administered in artificial adjuvants, like alum and complete Freund's adjuvant. For a number of responses, it seems that dendritic cells can serve as an "adjuvant." As shown in Table III, contact sensitivity, graft rejection, antibody formation, and responsiveness to protein antigens all have been induced by dendritic cells.

Intriguing findings were recently reported by Inaba *et al.* [26]. Dendritic cells were pulsed with protein antigens *in vitro* and then administered into the foot pads of mice. CD4⁺ T cells in the draining lymphoid tissue (brachial and popliteal lymph nodes) developed reactivity to the antigen that had been used to pulse the dendritic cell (Fig. 1). Several proteins were tested with similar results, indicating that dendritic cells can retain antigens for sufficient periods of time to find and prime specific T cells *in vivo*. Moreover, when F1

TABLE III

Immune Responses Induced by Dendritic Cells *in Vivo*

Contact sensitivity: TNP [94], FITC [95], oxalozone [90]
 Allograft rejection: MHC [71, 96-98], H-Y [99]
 Antibody formation: anti Id and anti-TMV [100]
 MHC-restricted, T cells: myoglobin, ovalbumin, conalbumin [26]

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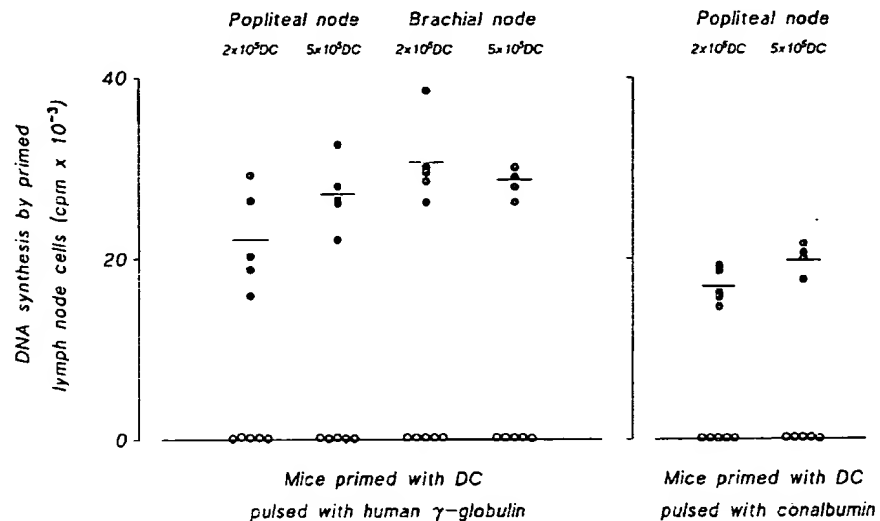


FIGURE 1 *In vivo* priming by antigen pulsed dendritic cells. Spleen adherent cells were cultured overnight in standard medium plus 0.1 mg/ml of human γ -globulin or conalbumin. After washing, the dendritic cells were purified by rosetting with antibody-coated red cells [31], and $2-5 \times 10^5$ antigen pulsed cells were injected into the rear or front foot pads of groups of 4-5 mice. The contralateral footpads were injected with dendritic cells that had not been pulsed with antigen. Five days later, the draining (closed) and contralateral (open), popliteal or brachial lymph nodes were taken to provide cell suspensions, which were cultured in microtest plates at 3×10^5 lymph node cells/well in triplicate in Click's medium supplemented with 0.5% mouse serum plus the corresponding antigen. DNA synthesis was measured after exposure to ³H-TdR at 4 uCi/ml at 56-72h. Note the reproducibility of the sensitization since each point is an individual animal. These findings are described more fully elsewhere [26].

mice were primed to parental strain dendritic cells, the T cells that were sensitized were predominantly restricted to the MHC of the priming dendritic cell. This provided evidence that the injected dendritic cells were not simply transporting antigens to be presented by host APC. Instead, the antigen-pulsed dendritic cells were directly presenting antigens to the T cell *in situ*. Extracorporeal pulsing of dendritic cells needs to be explored with other antigens, since this may provide a physiologic pathway for inducing T-dependent immunity.

Earlier in the review, we outlined the afferent and efferent limbs of primary T cell responses as they occur in tissue culture. How might these events transpire *in situ*? The experiments of Inaba *et al.* [26] emphasize an important feature of *in situ* priming. It occurs predominantly in the draining lymphoid organ, much as was discovered 65 years ago for the antibody response [101]. If antigen-pulsed dendritic cells were given in a hind foot pad, T cells were sensitized in the draining popliteal node but not in the contralateral node, or the inguinal and brachial nodes. Parallel findings were made if the injection was done in the front foot pad. It is known that stimulation of a local node leads to the release of large numbers of lymphoblasts into the efferent lymph [102], which are short-lived in the blood stream [103] probably because they home to inflammatory sites [104, 105]. This sequence of events would explain how dendritic cell-mediated induction of lymphoblasts in draining lymphoid tissue leads to the recruitment of those blasts to the original inflammatory site, where the efferent, T blast-APC interaction can occur.

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From: Rawlings, Stephen
Sent: Tuesday, September 23, 2003 4:57 PM
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Art Unit / Location: 1642/CM1,8E17
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Telephone Number: 305-3008
Application Number: 09821883

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Thank you.

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Dendritic Cells Pulsed with Protein Antigens In Vitro Can Prime Antigen-specific, MHC-restricted T Cells In Situ

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Summary

T cells recognize peptides that are bound to MHC molecules on the surface of different types of antigen-presenting cells (APC). Antigen presentation most often is studied using T cells that have undergone priming in situ, or cell lines that have been chronically stimulated in vitro. The use of primed cells provides sufficient numbers of antigen-reactive lymphocytes for experimental study. A more complete understanding of immunogenicity, however, requires that one develop systems for studying the onset of a T cell response from unprimed lymphocytes, especially in situ. Here it is shown that mouse T cells can be reliably primed in situ using dendritic cells as APC. The dendritic cells were isolated from spleen, pulsed with protein antigens, and then administered to naive mice. Antigen-responsive T cells developed in the draining lymphoid tissue, and these T cells only recognized protein when presented on cells bearing the same MHC products as the original priming dendritic cells. In contrast, little or no priming was seen if antigen-pulsed spleen cells or peritoneal cells were injected. Since very small amounts of the foreign protein were visualized within endocytic vacuoles of antigen-pulsed dendritic cells, it is suggested that dendritic cells have a small but relevant vacuolar system for presenting antigens over a several day period in situ.

The immunologic activity of T lymphocytes is directed to antigens presented by MHC products on the surfaces of other cells termed APC (1-4). While many cell types are capable of generating MHC-peptide complexes and presenting these to primed T cells, it is evident that the dendritic cell subset of APC greatly accelerates the early sensitization phase of the immune response. This has been noted in vitro with transplantation (5-7) and viral (8, 9) antigens, and in situ using contact (10, 11) and transplantation antigens (12-14). Nevertheless, experimental studies of T cell sensitization in situ to antigens that require processing typically utilize artificial adjuvants rather than viable APC. Whenever bulk spleen cells have been used as APC in the absence of adjuvants, it has not been possible to restrict the sensitization to antigens in association with MHC products of the injected cells (15, 16). Therefore presentation in situ likely involves host rather than injected APC.

Kurt-Jones et al. used B cells as APC in situ to reverse a lack of T-cell responsiveness in mice that had been suppressed chronically with anti- μ antiserum (17). The B cells, when given simultaneously with antigen in CFA, appeared to be capable of priming MHC-restricted T cells in some but not all cases. In contrast, Lassila et al. (18) reported that in a chicken system B cells could not present antigens to T cells in situ.

Likewise, in primary antibody responses to hapten-carrier conjugates in vitro, dendritic cells and not B cells are required as APC early in the immune response (19).

Here we have assessed the capacity of antigen-pulsed dendritic cells to sensitize the T cells of an unprimed individual. We find that specific priming occurs, and that the sensitized T cells are restricted to recognize antigen on the MHC products of the presenting dendritic cells. These results suggest that dendritic cells are "nature's adjuvant." They are capable of delivering exogenous antigens, most likely as complexes of peptides on surface MHC products, directly to naive T cells in situ.

Materials and Methods

Mice. BALB/C \times DBA/2 (C \times D2)F $_1$ (H-2 d), C3H/HeJ (H-2 k), and (C3H \times DBA/2)F $_1$ mice, 6-12 wk old and of both sexes, were purchased from The Trudeau Institute, Saranac Lake, NY.

Proteins. The antigens tested were sperm whale myoglobin, conalbumin, human gamma globulin, ovalbumin (Sigma Chemical Co., St. Louis, MO) and rhodamine-modified ovalbumin (Molecular Probes, Eugene, OR).

Culture Medium. For the preparation and antigen-pulsing of dendritic cells, the medium was RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 5% FCS, 50 μ M 2-ME,

and 20 $\mu\text{g}/\text{ml}$ gentamicin. For assessing T cell proliferative responses in vitro, the medium was Click's medium (Irvine Scientific, Santa Ana, CA) supplemented with 0.5% heat-inactivated mouse serum, 50 μM 2-ME, and 20 $\mu\text{g}/\text{ml}$ gentamicin.

Antigen "Pulsing" of APC. As will be evident in Results, it was necessary to expose fresh rather than cultured dendritic cells to a foreign protein to successfully charge these APC with antigen. Adherent cells from a low buoyant density fraction of spleen were prepared (20–22) and cultured overnight (12–18 h) in medium to which 0.1 mg/ml of protein antigen was added. After overnight culture, the dendritic cells were purified by rosetting most of the contaminants (macrophages, B cells) with antibody-coated erythrocytes (21, 22). In experiments to be reported elsewhere (23), we have found that fresh dendritic cells purified on a FACS can be charged with antigen with just a 3-h exposure. However, the longer overnight "pulse" (12–18 h) was used, since it simplified the purification of the dendritic cells and improved their APC function. To ensure that the dendritic cells had been successfully pulsed with antigen, we did conventional restimulation assays using primed lymph node T cells (see Results). The other APC populations that were pulsed with foreign proteins were resident peritoneal cells, maintained in Teflon beakers to reduce macrophage adherence, and unfractionated spleen cells. In some experiments, 4 mg of protein was given to mice intravenously or intraperitoneally, spleen dendritic cells were isolated as described (24), and these in vivo pulsed APC were readministered to naive mice.

Priming with Antigen-pulsed APC In Situ. Antigen-pulsed APC were washed at least three times in RPMI 1640 and administered in PBS at a dose of $2\text{--}60 \times 10^5$ cells in a volume of 25–40 μl into the fore or hind footpads. Generally, the antigen-pulsed APC were administered on one side, and the contralateral footpads served as the control. The control footpads were injected with APC that either had not been antigen-pulsed or were pulsed with a noncross-reacting protein (see Results). At varying times thereafter, but usually at day 5, the draining popliteal or brachial lymph nodes were removed, teased into a cell suspension, and challenged with antigen in vitro at 1–100 $\mu\text{g}/\text{ml}$. 3×10^5 cells were cultured in triplicate in flat-bottomed microtest wells (No. 25850; Corning Glassworks, Corning, NY). DNA synthesis was measured on the 3rd day after exposure to [^3H]TdR (specific activity, 6.0 Ci/mM) at 4 $\mu\text{Ci}/\text{ml}$ for 12–16 h. Unprimed lymph nodes never showed a response to the antigens we studied. The cells that responded by DNA synthesis in primed mice were shown to be primarily CD4⁺Thy-1⁺ cells by treatment with appropriate mAb and complement before the assay for DNA synthesis.

MHC Restriction of In Vivo Primed T Cells. C3H \times DBA/2 or A \times DBA/2 F₁ (Ia^b \times Ia^d) mice were primed with antigen-pulsed dendritic cells from either parental strain. 5 d later the draining lymph nodes were taken, and the cell suspensions were treated with mAb J11d anti-B cell and dendritic cell (22) and B21-2 anti-I-A plus rabbit complement (Pel-Freez Biologicals, Rogers, AR) to deplete lymph node APC. The cells were then cultured at 3×10^5 cells per microtest well in triplicate with graded doses of irradiated (1,000 rad ^{137}Cs) parental or F₁ spleen cells as APC with or without antigen. As will be evident in the Results, responsiveness was observed primarily when antigen was presented by the same parent that was used to prime the animals. To verify that the response was class II MHC-restricted, blocking studies with culture supernatants of anti-Ia mAbs were performed. The mAbs were B21-2 anti-Ia^d and 10-2.16 anti-Ia^b, both available at the American Type Culture Collection, Rockville, MD (TIB 229 and TIB 93, respectively).

Pinocytosis of Protein Antigens. Rhodamine-modified ovalbumin

proved to be a sensitive protein for visualizing pinocytosis by the weakly endocytic dendritic cells. Uptake was apparent after an overnight exposure to 0.1 mg/ml. Little or no uptake was evident at 0.02 mg/ml, or after a 2-h exposure, using fluorescence microscopy with a Zeiss Axiomat equipped for epifluorescence. The other tracers which we tested, which showed less and sometimes no uptake at the light microscopic level, were FITC-dextran, lucifer yellow, and horseradish peroxidase all at 0.1 mg/ml, the dose used to charge the APC with antigen. The positive control for active pinocytic activity was provided by resident macrophages in peritoneal washouts (see Results).

Results

Conditions for Pulsing Mouse Dendritic Cells with Protein Antigens In Vitro. We began with sperm whale myoglobin, for which prior studies had defined an immunodominant region in the H-2^d mouse corresponding to residues 106–118 (25, 26). Spleen adherent cells, which include dendritic cells, macrophages, and B cells, were cultured with or without native myoglobin overnight (16–24 h). The dendritic cells were then enriched by a standard method (21, 22) and tested for their capacity to stimulate myoglobin-primed T cells. As shown before (27), dendritic cells that had been cultured overnight without antigen were able to present peptide fragments, but presented native protein only weakly (Table 1, compare group 3 with groups 1 and 2). However, if the dendritic cells had been exposed to protein during the overnight culture, the antigen-pulsed APC vigorously stimulated the primed T cells (Table 1, group 4). Similar findings were made with other proteins (human gamma globulin, conalbumin, and ovalbumin), but we did not have active peptide fragments for these antigens. Similar results also were obtained if the dendritic cells were cultured for 2 d before use (Table 1, compare group 7 with groups 5 and 6). However, it was noted that the antigen pulse was best if given during the first rather than the second day of culture (Table 1, compare groups 8–10). Once pulsed, the dendritic cell maintained immunogenicity for at least a day in culture (Table 1, group 10). We conclude that freshly isolated dendritic cells can be successfully pulsed with a variety of soluble protein antigens in vitro, but that it is important to administer the antigen shortly after isolating the dendritic cells from the spleen.

Antigen-pulsed Dendritic Cells Sensitize T Cells In Situ. After exposure to one of four different proteins, as above, the dendritic cells were injected into the left hind foot pad; companion unpulsed dendritic cells were injected into the right side. The draining popliteal lymph nodes from groups of three to five mice were taken 5 d later and tested for responses in vitro to each of three different proteins. To avoid responses to FCS components, which were present during the time that the dendritic cells were pulsed with antigen, the lymph node cells were cultured in the presence of mouse rather than fetal calf serum.

For each protein, the lymph node draining the site of antigen-pulsed, dendritic cell deposition developed specific antigen responsiveness (Table 2). If we injected two populations of dendritic cells, each pulsed with different proteins, then the lymph node cells acquired reactivity to both antigens.

Table 1. Conditions for Pulsing Dendritic Cells with a Foreign Protein *In Vitro*

Group	Culture of dendritic cells before use as APC*	Antigen† during the APC-T coculture	DNA synthesis by antigen-primed T cells‡ to graded doses of dendritic cells§			
			3×10^4	10^4	3×10^3	10^3
1	0-24 h, no antigen	None	3.9	1.9	0.8	0.2
2	0-24 h, no antigen	Myoglobin	7.6	1.6	0.5	0.2
3	0-24 h, no antigen	Myopeptide	45.9	20.2	6.9	1.2
4	0-24 h, myoglobin	None	91.6	37.9	10.8	4.2
5	0-48 h, no antigen	None	3.6	1.4	0.7	0.3
6	0-48 h, no antigen	Myoglobin	0.3	0.6	0.3	0.3
7	0-48 h, no antigen	Myopeptide	39.2	18.2	4.7	0.7
8	0-48 h, myoglobin	None	81.8	27.3	8.9	3.2
9	0-24 without myo, 24-48 with myo	None	15.0	4.4	1.7	0.7
10	0-24 with myo, 24-48 without myo	None	88.5	27.6	9.8	4.0

* Low density spleen adherent cells, which are a partially enriched population of dendritic cells (20-22), were cultured for 1 or 2 d in medium supplemented with antigen (100 μ g/ml sperm whale myoglobin; Fluka) where indicated. After culture, contaminating macrophages and B cells were removed by rosetting with antibody-coated red cells.

† No antigen, myoglobin (5 μ M), or myoglobin peptide 105-118 (2.5 μ M), was added to the coculture of antigen-pulsed dendritic cells and myoglobin-primed T cells (below).

‡ Antigen-primed T cells were prepared as follows. Mice were primed with 5 μ m of myoglobin in CFA in the footpads. Brachial and/or popliteal lymph nodes were taken 5 d later and cell suspensions were prepared by teasing the nodes with forceps. The cell suspensions were cultured at 5×10^4 cells/well in 24-well trays in 1.5 ml Click's medium supplemented with 0.5% mouse serum, 2 mM L-glutamine, 50 μ M 2-ME, and 5 μ M myoglobin. 10 d later the contents of the flask were applied to Ficoll (Sigma) columns to collect viable lymphoblasts. By using T cells that had been expanded *in vitro*, rather than fresh lymph node cells, we could obtain populations that responded strongly to re-stimulation with specific antigen on dendritic cells, without the syngeneic mixed leukocyte reaction that typically elevates background DNA synthesis when lymph node cells are cultured with unpulsed dendritic cells.

§ 3×10^4 myoglobin-primed T blasts (see §) were cultured with graded doses of dendritic cells (see *) [3 H]TdR was added at 48-64 h to measure DNA synthesis. Data are mean cpm [3 H]TdR uptake $\times 10^{-3}$ for triplicate wells; standard deviations were <10% of the mean. The experiment was repeated once with similar results.

In these latter experiments, there was some development of antigen reactivity in the nondraining lymph node (Table 2; last pair of antigens).

In experiments that are not shown, the antigen-reactive lymph node cells were CD4⁺ T cells primarily, since >80% of the reactivity could be eliminated with either anti-Thy-1 or anti-CD4 mAb and complement. The sensitizing capacity of dendritic cells was reproducible, in that individual mice each gave responses of comparable magnitude.

In kinetic studies, the draining lymph node cells became responsive over a 5-d period to the specific protein that had been used to pulse the injected dendritic cells (Fig. 1). Antigen specificity was maintained at all time points, i.e., if the left foot pad had been injected with dendritic cells pulsed with human gamma globulin, the left popliteal node developed specific responsiveness to human gamma globulin but not to other proteins (Fig. 1, left). Likewise, the right popliteal node developed responsiveness to the protein used to pulse the dendritic cells that were injected into the right foot pad (Fig. 1, right).

When primed mice were rechallenged with antigen-pulsed dendritic cells but in a site distal to that used for the original priming (front vs. hind foot pad), the lymph node draining

this second site showed an accelerated or "memory" type response to the appropriately pulsed dendritic cells (Fig. 2). Responsiveness to antigen was apparent on the second day and virtually disappeared by the third.

Dendritic cells were also charged with a 2-h pulse of protein antigen *in vivo* as recently described (24). The dendritic cells were purified from the spleen using the FACS and injected into naive mice. Specific priming to the protein that originally had been given systemically to the dendritic cell donor was then observed (Table 3). We conclude that dendritic cells that had been pulsed with protein antigen *in vitro* or *in vivo* are capable of sensitizing CD4⁺ T cells from naive mice to that protein.

APC Requirements for Successful Priming *In Situ*. Dendritic cells were compared with two standard populations that have been used in many studies of antigen presentation *in vitro*. These were suspensions from spleen (a rich source of B lymphocytes) and peritoneal cavity (a rich source of macrophages and CD5⁺ B cells). The populations were pulsed with antigen for either 3 h or for 18 h in culture and administered in graded doses of 8×10^5 to 5×10^6 cells to the footpads of naive recipients (Table 4). Spleen cells were marginally effective, in that only the highest dose of 6×10^6 cells induced

Table 2. Dendritic Cells that Are Pulsed with a Protein Antigen *In Vitro* Specifically Prime Animals to that Protein *In Situ*

		DNA synthesis after challenge with						
Popliteal node	DC pulsed with:	No Ag	Conalbumin		Myoglobin		Hu × gamma globulin	
			100	10	100	10	100	10
<i>cpm × 10⁻³</i>								
Right	No Ag	0.4	0.7	0.4	0.7	0.6	3.2	2.0
Left	Conalbumin	0.3	<u>126.1</u>	<u>92.7</u>	1.3	0.8	3.4	0.9
Right	Myoglobin	0.2	0.4	0.2	<u>32.3</u>	<u>33.9</u>	1.4	0.9
Left	No Ag	0.2	0.5	0.3	0.3	0.4	1.9	0.1
Right	HGG	0.2	0.5	0.3	0.2	0.4	<u>27.4</u>	<u>18.0</u>
Left	No Ag	0.4	0.8	0.7	0.7	0.4	2.6	2.3
Right	Conalbumin + Myoglobin	0.5	<u>58.7</u>	<u>18.5</u>	<u>40.0</u>	<u>42.7</u>	3.7	2.4
Left	HGG	0.2	0.3	0.2	0.3	0.3	<u>18.6</u>	<u>11.6</u>
Right	Conalbumin	0.7	<u>99.6</u>	<u>79.5</u>	2.2	1.2	<u>19.6</u>	<u>8.7</u>
Left	Myoglobin + HGG	0.7	<u>8.7</u>	<u>4.5</u>	<u>73.8</u>	<u>62.9</u>	<u>119.4</u>	<u>104.6</u>
Right/left	No DC	0.1	0.2	0.2	0.2	0.2	0.6	0.3

Low density spleen adherent cells were cultured for 1 d with or without antigen, after which macrophages and B cells were depleted (21, 22). 2×10^5 dendritic cells were injected in the foot pads of groups of four mice. 5 d later, the draining popliteal nodes were taken and cell suspensions were prepared by teasing with fine forceps. 3×10^5 primed lymph node cells were cultured in flat-bottomed microtiter wells without antigen, or with the indicated antigens at 100 or 10 $\mu\text{g/ml}$. [^3H]TdR was added at 44–60 h to measure DNA synthesis.

Table 3. Priming of Antigen Specific Lymph Node T Cells Using Dendritic Cells that Have Been Pulsed with Antigen *In Vitro* or *In Vivo*

Dendritic cells [2×10^5] used to prime brachial nodes	DNA synthesis of primed lymph node boosted in vitro with*					
	No Ag	Conalbumin ($\mu\text{g/ml}$)			BSA	OVA
		100	10	1	100	100
		$\text{cpm} \times 10^{-1}$				
In vitro pulse†						
Myoglobin	0.1	0.1	—	—	0.9	—
Conalbumin	0.4	155.4	148.8	119.3	3.3	1.1
Conalbumin pulse + 25 μg soluble Ag in paw	0.4	161.8	145.3	124.1	4.8	0.3
In vivo‡						
Conalbumin	0.3	68.7	45.5	22.1	1.5	1.0

* 5 d after priming with dendritic cells in the front footpad, brachial lymph node cells were prepared and restimulated in culture with the indicated antigen. DNA synthesis was measured on the third day.

† Low density spleen adherent cells were pulsed with 100 $\mu\text{g/ml}$ of protein overnight. Dendritic cells were then purified by depleting FcR⁺ cells (21, 22).

‡ Mice were given 4 mg conalbumin i.v. 2 h later, the spleens were taken, and dendritic cells were isolated using the FACS and the N418 mAb to murine CD11c (24). N418 primarily reacts with dendritic cells in mouse spleen (23).

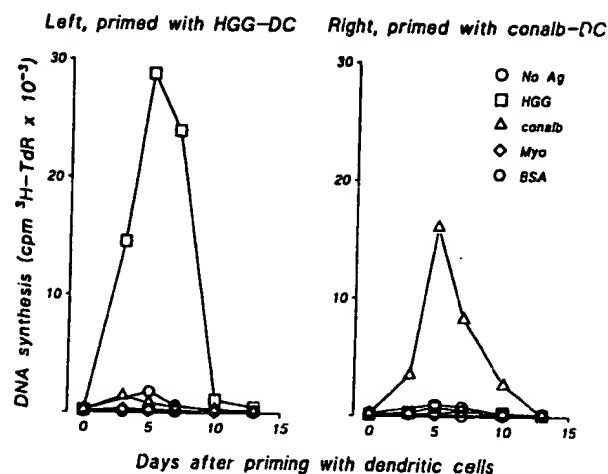


Figure 1. Kinetics of the primary response to antigen-pulsed dendritic cells. Low density spleen adherent cells were cultured with or without 100 μ g/ml of the indicated proteins for 24 h. The cells were washed and 3×10^5 FcR⁻ dendritic cells were injected into each foot pad. At the indicated time points, the draining popliteal and brachial lymph nodes were taken and cultured with the indicated proteins at 100 μ g/ml in Click's medium supplemented with 0.5% mouse serum. DNA synthesis was measured on the third day. Lymph node cells that were primed with dendritic cells that had not been pulsed with antigen did not exhibit an antigen response (not shown; but see Table 2). The experiments were repeated twice with similar results.

a low level of responsiveness in the draining lymph node. Peritoneal cells were ineffective at all doses. Inocula of 2 and 5×10^5 dendritic cells had similar effects in situ, and the minimum dose capable of inducing some responsiveness was $3-8 \times 10^4$ dendritic cells (Table 4).

MHC Restriction of T Cells Primed by Antigen-pulsed Dendritic Cells. The finding that antigen-pulsed dendritic cells could prime naive animals to that antigen could be explained by a unique ability of dendritic cells to stimulate T cells directly in vivo, or alternatively, to transport antigens that were presented subsequently by host APC. The two possibilities could be distinguished by assessing whether the T cell sensitization process was restricted to antigens presented on the injected vs. host dendritic cells. We primed F₁ mice with antigen-pulsed dendritic cells from either parental strain and tested if the primed F₁ T cells could only be boosted with spleen APC from the original parent. It is known that most clones of T lymphocytes in an F₁ animal are restricted to antigens presented by one or the other parental MHC (28, 29). We used A \times DBA/2 (H-2^b \times H-2^d) or C3H \times DBA/2 (H-2^k \times H-2^d) F₁ recipients and primed with antigen-pulsed dendritic cells from each parental strain. 5 d later the F₁ lymph node cells were isolated, depleted of endogenous APC by treatment with anti-Ia and J11d mAb and complement, and challenged with APC from the F₁ or from either parent.

The F₁ T cells responded vigorously to antigen rechallenge in vitro with F₁ APC (Table 5). If parental strain APC were used, the rechallenge was far more effective with APC from the same parental strain that was used to sensitize the local node (Table 5). To show that the responses were re-

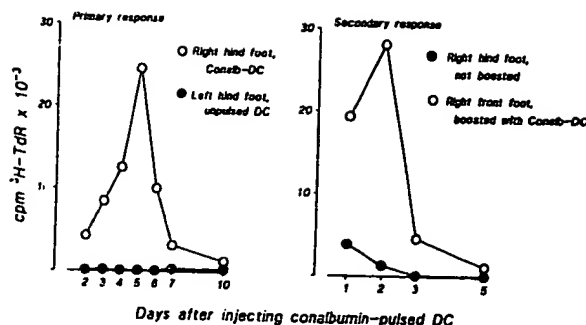


Figure 2. Secondary responses in mice that had been primed with antigen-pulsed dendritic cells. Conalbumin-pulsed dendritic cells (DC) were injected into the right hind foot pad as in Fig. 1, while the left hind foot pad received dendritic cells that were not pulsed with conalbumin. At the indicated time points (left), the draining lymph nodes were taken and boosted with protein antigen in vitro. ¹ that the primary response peaks at day 5 and subsides by day 7-9, as in Fig. 1. Companion groups of mice were then boosted on day 12 with antigen-pulsed DC, but in the right front foot pad to look for a secondary response (right). The experiment was repeated twice with similar results.

stricted to the class II MHC molecules of the sensitizing dendritic cells, we verified that a mAb to I-A^d blocked the restimulation of F₁ T cells primed with H-2^d APC but not with H-2^b APC, while mAb to I-A^k blocked presentation by H-2^k APC but not H-2^d APC (Table 6).

Visualization of Antigen Uptake in Dendritic Cells. Since one pathway for antigen presentation likely involves endocytosis of the foreign protein followed by proteolysis and formation of peptide-MHC complexes (4), we monitored the extent to which dendritic cells could accumulate a protein that we could visualize, rhodamine-modified ovalbumin. When dendritic cells were pulsed overnight in 0.1 mg/ml of protein, it was evident that each cell had a small number of fluorescent granules, usually close to the nucleus (Fig. 3, left). Macrophages in contrast were much more heavily labeled after exposure to rhodamine-ovalbumin (Fig. 3, right). These results suggest that the strong APC function of dendritic cells in situ is associated with the accumulation of only small amounts of the foreign protein.

To verify that the rhodamine tracer that was being visualized in the above experiments was in fact relevant to immunogenicity in situ, we charged dendritic cells with rhodamine-modified ovalbumin and administered the cells to mice. 5 d later cells from the draining lymph node were tested for antigen responsiveness. Interestingly, responsiveness developed in a dose-dependent manner (Fig. 4, left) but it was specific for the conjugate rather than the free ovalbumin carrier (Fig. 4, right). Therefore, the rhodamine group that we were monitoring in the uptake studies above (Fig. 3) was relevant to the antigen-specific sensitization that was occurring in situ.

Discussion

Several features of dendritic cells help explain the sensitizing function of these APC in situ (30). One is their capacity to

Table 4. Dendritic Cells Are Specialized to Present Antigens In Vivo

Exp.	Myoglobin-pulsed APC used to prime in vivo		Proliferation of primed lymph node cells with			
			No Antigen	Myoglobin		Conalbumin 100 µg/ml
				5 µm	0.5 µm	
1	Spleen cells	5 × 10 ⁶	0.1	5.6	0.8	0.2
		2 × 10 ⁶	0.1	2.1	0.2	0.2
		8 × 10 ⁵	0.1	0.1	0.1	0.2
	Dendritic cells	5 × 10 ⁵	0.1	32.5	28.1	0.3
		2 × 10 ⁵	0.1	29.9	17.6	0.1
		8 × 10 ⁴	0.1	4.2	0.6	0.1
	Peritoneal cells	2 × 10 ⁶	0.1	0.1	—	0.1
		8 × 10 ⁵	0.1	0.1	0.1	0.1
	None	—	0.1	0.1	0.1	0.1

	Conalbumin-pulsed APC used to prime in vivo		Proliferation of primed lymph node cells with				
			No Antigen	Conalbumin (µg/ml)			Ovalbumin 100
				100	10	1	
2	Spleen cells	2 × 10 ⁶	0.3	9.8	6.3	2.0	0.2
		1 × 10 ⁶	0.3	2.1	0.5	0.2	0.2
		5 × 10 ⁵	0.3	0.3	0.3	0.2	0.2
		2.5 × 10 ⁵	0.3	0.3	0.2	0.2	0.2
	Dendritic cells	5 × 10 ⁵	0.3	56.3	54.2	19.6	0.3
		2.5 × 10 ⁵	0.3	47.2	40.1	12.1	0.3
	Peritoneal cells	2 × 10 ⁶	0.2	0.4	0.3	0.3	0.2
		1 × 10 ⁶	0.2	0.3	0.3	0.2	0.2
		5 × 10 ⁵	0.2	0.3	0.2	0.2	0.2
		2.5 × 10 ⁵	0.2	0.2	0.2	0.2	0.2

Three different populations of APC were exposed to 100 µg/ml of myoglobin for 16 h (Exp. 1) or conalbumin (Exp. 2), washed, and administered into the footpads of naive mice at the indicated doses. 5 d later the draining lymph nodes were taken, and the cell suspensions were restimulated with antigens as shown. Data are cpm [³H]TdR uptake × 10⁻³ at 48–60 h. Data are not shown for APC that were not pulsed with antigen before administration to mice, since the proliferative responses were all 0.3 or less.

capture and retain antigens, a feature that seems to be short-lived in culture (Table 1) (27). Another is their capacity to form stable conjugates with resting, antigen-specific T cells and induce the development of functional T lymphoblasts (31–33). A third feature is the ability to home via the lymph and the blood (34–36) to the T-dependent areas of lymphoid organs. These functions together should allow the antigen-pulsed dendritic cell to select clones of specific T cells from the recirculating pool (37).

The number of dendritic cells that were effective in our experiments, 1–3 × 10⁵, is not large when other variables

are considered. The efficiency with which these cells leave the injection site and home to the draining lymph node may be very small, since Kupiec-Weginski et al. (34) reported that only 1–2% of a dose of ¹¹¹In-labeled dendritic cells were retained within the draining lymph node. This means that a few thousand dendritic cells may carry out the sensitization of T cells reported here. A similar conclusion was reached in studies of pancreatic islet transplantation. There, a maximum of 2,000–4,000 dendritic cells seemed responsible for sensitizing mice across an MHC barrier (13). The efficiency of these APCs likely relates to the fact that they home to

Table 5. Primed F₁ T Cells Are Restricted to the Parental Strain of the Dendritic Cells Used in Priming

Exp.	Spleen APC used to boost F ₁ T cells <i>in vitro</i>		DNA synthesis by F ₁ T cells			
			Primed with Ia ^b DC		Primed with Ia ^d DC	
	Strain	Dose	- Ag	+ Ag	- Ag	+ Ag
<i>cpm × 10⁻³</i>						
A: A × DBA/2 F ₁ T cells	A × DBA/2	3 × 10 ⁵	0.6	11.4	0.3	11.8
	[Ia ^{bcd}]					
	A	3 × 10 ⁵	0.5	22.4	0.1	0.1
	[Ia ^b]	5 × 10 ⁵	0.8	29.6	0.3	0.6
	BALB/C	3 × 10 ⁵	0.3	2.7	0.2	22.9
	× DBA/2	5 × 10 ⁵	0.4	3.6	0.6	65.4
B: C3H × DBA/2 F ₁ T cells	C3H × DBA/2	1.25 × 10 ⁵	0.2	14.7	0.3	58.2
		[Ia ^{bcd}]				
		2.50 × 10 ⁵	0.6	21.8	0.5	73.0
	BALB/C	5 × 10 ⁵	1.1	25.0	3.4	67.1
		1.25 × 10 ⁵	0.2	0.5	0.2	46.7
	× DBA/2	2.50 × 10 ⁵	0.8	1.3	0.9	76.7
		[Ia ^{bcd}]				
		5 × 10 ⁵	2.0	2.2	2.5	69.0
	C3H	1.25 × 10 ⁵	0.1	13.3	0.4	0.8
		[Ia ^b]				
		2.50 × 10 ⁵	0.4	31.6	1.2	1.4
		5 × 10 ⁵	1.8	26.2	2.9	3.4

F₁ mice (Ia^{bcd}) were primed with 3 × 10⁵ dendritic cells (DC) that had been pulsed with 100 µg/ml of conalbumin. The dendritic cells were from either parental strain. 5 d later, the lymph nodes were taken and cell suspensions were prepared by teasing with fine forceps. These suspensions were treated with B21-2 anti-Ia, and J11d mAb and complement to deplete endogenous APC, and then plated at 3 × 10⁵ cells in flat-bottomed microtiter wells in Click's medium with 0.5% mouse serum. Irradiated spleen cells (1,500 rad ¹³⁷Cs) from either parental strain, or the F₁ strain, were then added at the indicated doses as a source of APC. Conalbumin was (+ Ag) or was not (- Ag) added to the cultures at 100 µg/ml. DNA synthesis was measured at 44-60 h.

Table 6. Priming by Antigen-pulsed Dendritic Cells *In Vivo* is MHC-restricted

Strain of dendritic cells used to prime	Strain of spleen used to present antigen in vitro [†]	DNA synthesis in presence of: [‡]			
		no Ab	αCD4	αIa ^b	αIa ^d
		<i>cpm</i> × 10 ⁻³			
C3H × D2 F ₁ T cells*	C3H, H-2 ^k	55.9	8.5	11.7	42.6
	C3H, H-2 ^k	12.5	1.1	3.2	10.1
BALB/C × DBA/2, H-2 ^d	BALB/C × DBA/2, H-2 ^d	155.5	12.0	128.0	23.8
	C3H × DBA/2	86.2	9.0	41.7	12.9
	(H-2 ^{kcd})				

* 2 × 10⁵ dendritic cells, pulsed with conalbumin, were injected into C3H × DBA/2 F₁ (H-2^k × H-2^d) mice. 5 d later, lymph nodes were treated with antibodies to Ia and J11d + complement to deplete endogenous APC. 3 × 10⁵ T cells were then cultured per well. The experiment was repeated once with similar results.

† 3 × 10⁵ spleen cells from the indicated strains were irradiated (¹³⁷Cs, 1,000 rad) and added to the primed T cells.

‡ DNA synthesis was measured by adding [³H]TdR at 66-72 h. Conalbumin was added in all the experimental cultures that are shown, since proliferation in the absence of antigen was <1 × 10³ cpm. The mAbs tested for blocking activity were GK1.5 αCD4, 10-12.16 αI-A^b, and B21-2 αI-A^d.

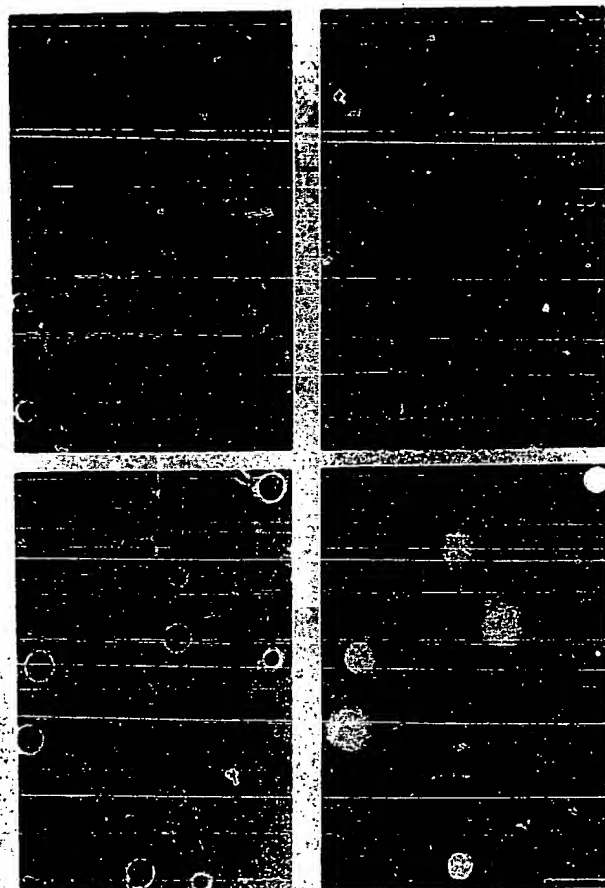


Figure 3. Detection of endocytic activity by antigen-pulsed dendritic cells. Low density spleen adherent cells were cultured overnight in 0.1 mg/ml of rhodamine-modified ovalbumin. The FcR⁺ dendritic cells were enriched and attached to glass slides coated with poly-L-lysine (21). The dendritic cells (top) were uniform in cytologic appearance (left) and each contained small numbers of rhodamine-labeled granules. In parallel, peritoneal cells were also exposed RITC-OVA. The large macrophages (bottom, arrows), as verified by strong indirect immunofluorescent staining with mAb to the M-1/70 CD11b antigen, were intensely labeled with the endocytic tracer (right). $\times 300$.

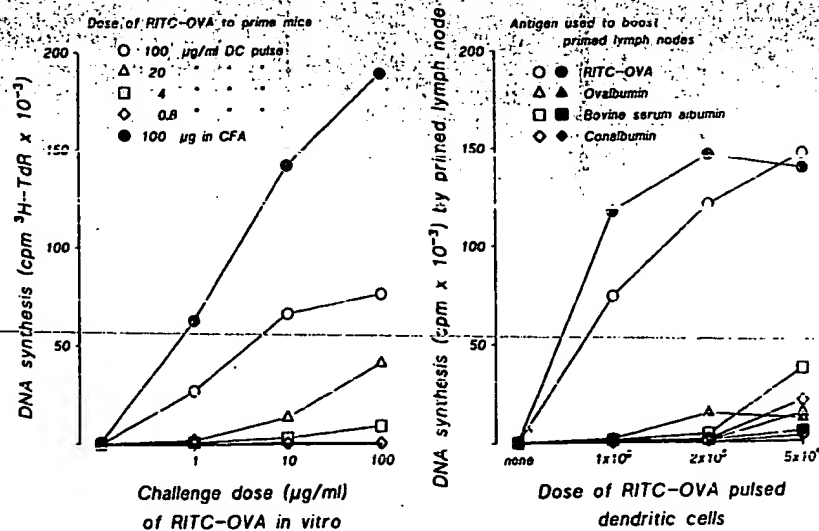


Figure 4. Rhodamine-ovalbumin (RITC-OVA)-pulsed dendritic cells prime mice in situ. Low density spleen adherent cells were exposed to graded doses of RITC-OVA (left) or to 0.1 mg/ml RITC-OVA (right) for 16 h, and then the dendritic cells were purified and injected at a dose of 3×10^5 into fore and hind foot pads (left) or in graded doses (right) into groups of three mice. 5 d later the draining lymph nodes were taken and boosted with graded doses of RITC-OVA (left) or RITC-OVA vs. other proteins (right). Only the data with 100 $\mu\text{g/ml}$ protein are shown on the right, with the popliteal and brachial nodes in open and closed symbols, respectively. [^3H]TdR uptake was measured at 48–60 h.

an optimal site in the lymphoid tissue and are each capable of binding and activating large numbers of T cells even with relatively small amounts of ligand on the dendritic cell surface (38).

Since the endocytic apparatus may provide an important route for the processing and presentation of exogenous antigens (4), it is of interest that the poorly endocytic dendritic cell (Fig. 3) is nonetheless extremely active in antigen presentation. This suggests to us that the endocytic apparatus of dendritic cells may be specialized to present antigens, whereas in macrophages, the bulk of the endocytic activity results in antigen clearance and degradation (39). In the experiments described here, it is formally possible that non-dendritic cells in the adherent spleen preparation were processing and regurgitating peptides onto dendritic cell MHC molecules, but in a recent study (24), we presented evidence that such a phenomenon is undetectable in our cultures. In addition, we have also prepared highly enriched populations of dendritic cells fresh from spleen, using the FACS, and found them to be potent APC for priming T cells in situ (23).

Once CD4^+ or CD8^+ T cells pass a control point that involves activation by dendritic cells, the sensitized T cells efficiently interact with other types of APC to carry out various effector functions that are critical for T cell-mediated immunity. For example, CD4^+ T blasts that are induced by dendritic cells can interact in an antigen-specific way to make B lymphocytes grow and respond to B cell stimulating factors (40), and to make macrophages synthesize IL-1 (41, 42). CD8^+ T blasts that are induced by dendritic cells can kill other APC as targets (43, 44). These "effector" aspects of the immune response may be restricted to inflammatory sites in situ, given the evidence that sensitized T cells that are produced in lymphoid tissues emerge into the lymph (45) and can move via the blood stream to inflammatory sites (46, 47). It is of interest that dendritic cells are not known to have any effector or antigen elimination functions, in contrast to other APC such as B cells and macrophages which can re-

lease antibody or kill microorganisms. The specialized role of dendritic cells seems to be to sensitize T lymphoblasts, which then interact with other APC.

Extracorporeal pulsing of antigens onto dendritic cells may provide a new approach to immunization *in situ*, a goal that previously could be approached only empirically with adjuvants. By using dendritic cells as a natural adjuvant, one has

an opportunity of having the APC select those epitopes on a complex antigen that can be presented by a given individual's MHC products. This strategy provides a physiologic selection of immunogen that may serve as an important alternative to the empirical search for immunogenic peptides that is being pursued so actively in recent years.

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Thank you..

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ORIGINAL ARTICLE

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Humoral and cellular responses raised against the human HER2 oncoprotein are cross-reactive with the homologous product of the *neu* proto-oncogene, but do not protect rats against B104 tumors expressing mutated *neu*

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Abstract The *neu* proto-oncogene encodes a plasma membrane protein belonging to the epidermal growth factor receptor family. The cell line B104, derived from a BDIX rat neuroblastoma, carries a point mutation in *neu*, and forms a tumor when injected into these rats. The human homologue of the *neu* oncogene (here called *HER2*) is overexpressed in certain types of cancer. Rats were immunized with *HER2* protein (*HER2*) to investigate a possible cross-reaction between the homologous proteins which could protect them against subsequent inoculation with B104. Specific antibody in the serum was measured by cell-based enzyme-linked immunosorbent assay and fluorescence immunocytochemistry, and delayed-type hypersensitivity by an ear assay. Sera from animals immunized with the *HER2* extracellular domain (*HER2*-ECD) reacted with both *HER2*- and *neu*-expressing cells. In the ear assay, a significant cellular response to both *HER2*-ECD ($P < 0.05$) and *neu* protein ($P < 0.001$) was observed in *HER2*-ECD-immunized rats. However, the growth of B104 tumors in rats was not affected by preimmunization with *HER2*-ECD. The results indicate that an autoreactive immune response to *neu* was induced by immunization with *HER2*-ECD, but was too weak to affect the growth of the *neu*-bearing tumor.

Key words *HER2* · *neu* · Immunization · Tumor antigen · B104 tumor

Introduction

The *neu* proto-oncogene encodes a 185-kDa cell-surface glycoprotein belonging to the epidermal growth factor receptor family. A mutated form of this gene (*neu**), isolated from neuroblastoma cells (B104) that had formed in the offspring of BDIX rats exposed *in utero* to ethylnitrosourea [24], encodes a protein that differs from the normal cellular product only by a single amino acid substitution (valine to glutamic acid) in the transmembrane region [1]. Transfection of this oncogene induces neoplastic transformation [21] in NIH 3T3 cells, which are then capable of forming tumors [9] and metastases [32], when injected into nude mice. The human homologue of *neu*, named *HER2* [4] or *c-erbB-2* [31], shows more than 90% homology with the rat oncogene. However, in contrast to *neu*, the transforming potential of *HER2* appears to be due to gene amplification and overexpression, rather than mutation [18, 25]. *HER2* amplification, observed in adenocarcinomas of the breast and other organs, was reported to correlate with increased metastasis and a decrease in patient survival time [27].

As this oncoprotein (185^{neu/HER2}) is expressed at the cell surfaces and possesses a large ectodomain, there has been considerable interest in it as a possible target for cancer immunotherapy [26]. Studies *in vitro* have shown that down-modulation of p185 expression by specific monoclonal antibodies, leads to the loss of the malignant phenotype in transfected NIH 3T3 cells either expressing *neu** [8] or overexpressing *HER2* [16]. Experiments in nude mice showed that fibrosarcomas induced by the injection of B104.1.1 cells (NIH 3T3 cells transfected with *neu**) regressed when treated with an anti-(*neu* protein monoclonal antibody [9]. This antibody also inhibited the anchorage-independent growth of B104 neuroblastoma cells in agar [8], and tumor formation by these cells when injected into syngeneic BDIX rats [9]. Bernards et al. [2] were able to protect mice against tumor challenge with B104.1.1 cells by previous immunization of the animals with a vaccinia virus recombinant expressing the extracel-

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lular domain of the *neu* protein. However this immunization protocol did not protect BDIX rats against tumor challenge with B104 cells.

As the *neu* protein is an autoantigen in the rat, the latter result is not surprising. It would be necessary to break tolerance in order to induce an anti-(*neu* protein) response in such animals. Immunization of animals such as Lewis rats with heterologous spinal cord leads to a more acute form of autoimmune encephalomyelitis than when the autologous protein is used, and it has been assumed for many years now that this phenomenon is due to foreign components in heterologous cord providing an adjuvant effect [22]. *HER2* (human) and *neu* (rat) show 90% sequence homology. It is possible, therefore, that through manipulation of the rat immune response to the heterologous *HER2* protein, a B or T response to the autologous rat protein may be induced.

The purpose of this study was to elicit an immune response to *HER2* protein in BDIX rats, and determine whether a possible cross-reactive response to the *neu* protein was able to protect animals against tumor challenge with B104 cells.

Materials and methods

Rats

Breeding pairs of BDIX rats were obtained from the National Cancer Institute animal colony (Frederick, Md.). These rats were subsequently bred through brother-sister matings at the animal facilities of the Venezuelan Institute for Scientific Research (IVIC). Female animals, approximately 8 weeks old, were used in the present experiments.

Cell lines

Transfected NIH 3T3 cells overexpressing the native form of *neu* (G/DHFR-8), and rat neuroblastoma cells carrying the mutated form of *neu* (B104), were a kind gift from Prof. R. A. Weinberg, Whitehead Institute for Biomedical Research, Cambridge, Mass., USA. The 3T3 cells were cultured in Dulbecco modified Eagle medium (DMEM) containing 10% calf serum (Gibco laboratories, Grand Island, N.Y. USA), to which 0.3 μ M methotrexate (Sigma Chemical Co., St. Louis, Mo., USA) was added in the case of the G/DHFR-8 cells. B104 rat cells were grown in DMEM containing 10% fetal bovine serum (FBS, Gibco Laboratories, Grand Island, N.Y., USA). The human mammary tumor line, SKBR-3 (the kind gift of Dr. Manuel Rieber, IVIC, Venezuela), which overexpresses *HER2*, was cultured in a 1:1 mixture of DMEM and Ham nutrient mixture F-12, containing 10% fetal bovine serum.

Antibodies

The monoclonal antibodies 4D5 and 3E8, are specific for different epitopes on the extracellular domain of the *HER2* oncoprotein (*HER2*-ECD), and were a gift from Genentech Inc., San Francisco, Calif., USA [10]. The monoclonal antibody 7.16.4 recognizes the rat *neu*-oncogene-encoded p185 molecule [7], and was a gift from Dr. M. Greene, Univ. Pennsylvania, Pa., USA. Two control antibodies were used in the enzyme-linked immunosorbent assay (ELISA): HB55, a pan anti-HLA-DR monoclonal antibody, and OKT8.

Immunization with *HER2*-ECD

Purified recombinant human *HER2*-ECD (residues 1–624) was a gift from Genentech Inc., San Francisco, Calif., USA. Female BDIX rats were immunized subcutaneously (s.c.) with 25 μ g *HER2*-ECD in

complete Freund's adjuvant (CFA), then boosted 15 days later with the same amount of *HER2*-ECD in incomplete Freund's adjuvant (IFA). Control animals were injected with phosphate-buffered saline (PBS)/CFA and PBS/IFA.

Tumor inoculation and growth

Viable B104 cells (2.5×10^6 in 0.1 ml PBS) were injected s.c. into the hind leg of female rats. Growing tumors were measured with vernier calipers twice weekly in three dimensions, and the product was taken as the tumor volume.

Serum antibody responses to *neu/HER2* protein

Blood was obtained by cardiac puncture from control or *HER2*-ECD-immunized animals, 15 days after the second immunization. The serum obtained from this blood was stored at -20°C till used. SKBR-3 or G/DHFR-8 cells were grown to near confluence in 96-well microtiter plates (Immulon, Dynatech Laboratories Inc., Chantilly, Va., USA). The cells were then fixed with glutaraldehyde and blocked according to the method of Carroll [3]. Sera were serially diluted in PBS containing 0.1% Tween-20 and 0.2% bovine serum albumin, then 100- μ l aliquots were placed in wells and incubated for 1 h at 37°C . Control monoclonal antibodies were added at a concentration of 5 μ g/ml. After three washes with PBS containing 0.1% Tween-20 (PBS/Tween), wells were incubated with a horseradish-peroxidase-conjugated second antibody, a goat anti-(rat IgG) monoclonal antibody for the rat sera, and a goat anti-(mouse IgG) antibody for the control mouse monoclonal antibodies. The goat anti-(rat Ig) and anti-(mouse Ig) antibodies were conjugated to horseradish peroxidase (all from Sigma Chemical Co., St. Louis, Mo., USA) by the periodate method [15]. Samples of 100 μ l at a 1:40 dilution in PBS/Tween were added to each well and incubated for 1 h at 37°C . The wells were washed three times with PBS/Tween, then 100 μ l *o*-phenylenediamine/ H_2O_2 substrate was added. The reaction was stopped after 15–20 min with H_2SO_4 and the absorbance read at 490 nm.

Immunofluorescence

Subconfluent cells were harvested with PBS containing 2 mM EDTA (PBS/EDTA), washed once by centrifugation with PBS, then counted. Aliquots of 5×10^5 cells were placed in tubes then washed once with 500 μ l working buffer of PBS containing 2% FBS and 0.1% sodium azide. The cells pellets were then resuspended in 100 μ l 1/100 dilution of serum from immunized or control animals in working buffer on ice for 30 min. The cells were washed once with the same buffer, then resuspended in 50 μ l 1/40 dilution of fluorescein-isothiocyanate (FITC)-conjugated goat anti-(rat Ig) for 30 min on ice. The antibody was conjugated to FITC (Sigma Chemical Co., St. Louis, Mo., USA) by a standard method [15]. The cells were then evaluated by fluorescence microscopy.

Radiometric ear assay

Delayed-type hypersensitivity to *HER2/neu* protein was quantified by a radiometric ear assay [28]. SKBR-3, G/DHFR-8 and NIH 3T3 cells were harvested with PBS/EDTA, then washed twice with PBS. Thirty days after the second immunization with *HER2*-ECD, rats were injected in the pannus of the left ear with 2.5×10^6 SKBR-3 cells in 100 μ l PBS, and in the right ear with 100 μ l PBS. In those animals that received 2.5×10^6 G/DHFR-8 cells in the left ear, 2.5×10^6 NIH 3T3 cells were injected into the right ear. Immediately prior to the ear injections, the animals were injected s.c. with 0.25 μ Ci/g body weight of tritiated thymidine. Ears were removed after 48 h and prepared for liquid scintillation counting as follows. Ears were placed in vials with 4 ml NCS-II tissue solubilizer (Amersham Corp., Arlington Heights, Ill., USA) and 1 ml H_2O , then left to digest completely at 37°C (24–48 h). Aliquots of 1 ml were mixed with 12 ml scintillation fluid (Aqasol-2, Dupont, Boston, Mass., USA), then counted in a liquid scintillation counter. The radiometric ear index represents the ratio of thymidine incorporation into the two ears (left/right).

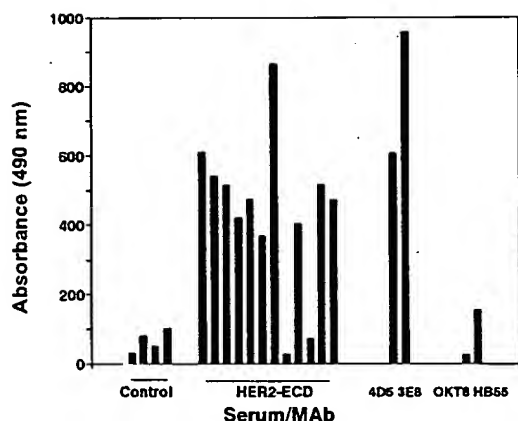


Fig. 1 Anti-HER2 antibodies in the serum of rats immunized with *HER2* protein extracellular domain (*HER2-ECD*) as measured by enzyme-linked immunosorbent assay (ELISA). Sera from control and *HER2-ECD*-immunized rats were assayed at a 1:1000 dilution with an ELISA in which the wells were coated with *HER2*-overexpressing SKBR-3 cells. Monoclonal antibodies 4D5, 3E8 (anti-*HER2*-protein) and OKT8, HB55 (irrelevant) were added at 5 μ g/ml

Statistical analysis

As the radiometric ear assay results showed a non-normal distribution, the geometric mean was taken. The range was calculated from the antilogarithm of the logarithmic mean and standard deviation $[(x-SD)-(x+SD)]$. Groups were compared using the Mann-Whitney test.

Results

Out of 12 BDIX rats immunized with *HER2-ECD*, 10 showed a humoral response to this protein as measured

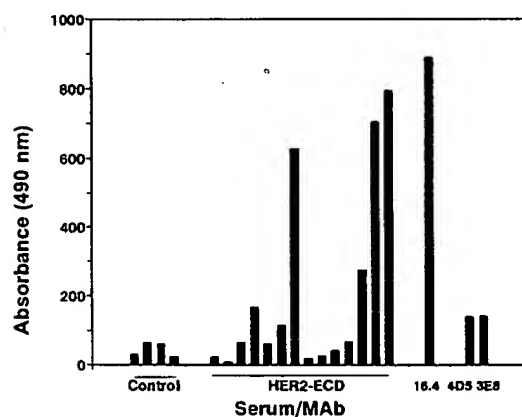


Fig. 2 Anti-*neu* protein) antibodies in the serum of rats immunized with *HER2-ECD* as measured by ELISA. Sera from control and *HER2-ECD*-immunized rats were assayed at a 1:1000 dilution with an ELISA in which the wells were coated with *neu*-overexpressing G/DHFR-8 cells. Monoclonal antibodies 16.4 (anti-*neu*-protein) and 4D5, 3E8 (anti-*HER2*-protein/control) were added at 5 μ g/ml

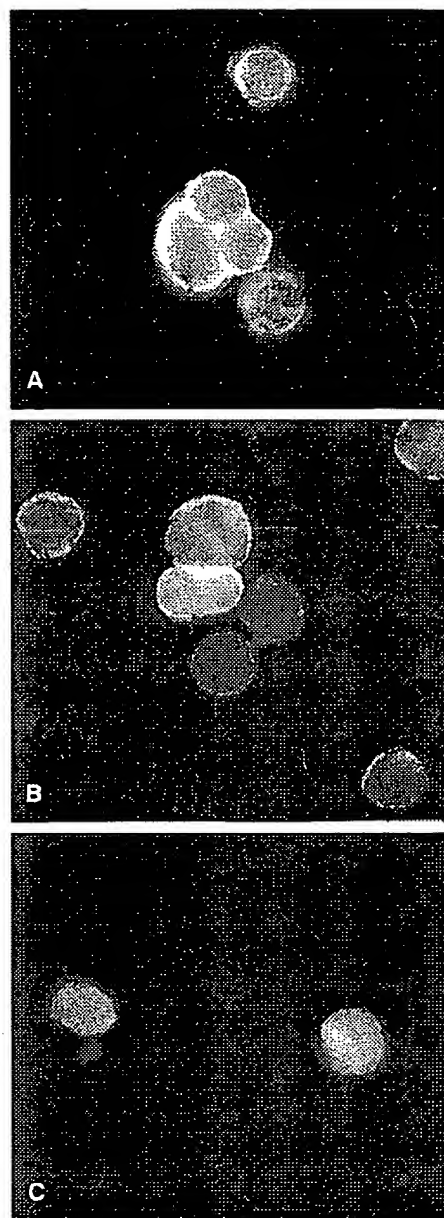


Fig. 3 A–C Detection of anti-*HER2* protein) and anti-*neu* protein) antibodies by immunofluorescence. SKBR-3, NIH 3T3 and G/DHFR-8 cells were harvested without trypsin, then incubated with either rat serum (1/100) from *HER2-ECD*-immunized or control animals. Fluorescein-isothiocyanate-conjugated goat anti-(rat Ig) antibody was added to reveal antibody binding. A SKBR-3 cells + immune serum. B G/DHFR-8 cells + immune serum. C 3T3 cells + immune serum

by ELISA, with titres above 1:10⁵ in most animals. Anti-*HER2* protein) antibodies were not detectable in the four control animals. Figure 1 compares the absorbances obtained at a serum dilution of 1:1000, together with those for two anti-*HER2* monoclonal antibodies (4D5, 3E8), and two irrelevant monoclonal antibodies (HB55, OKT8).

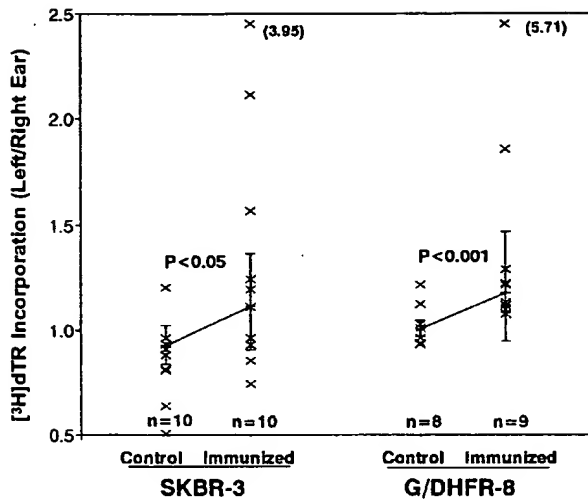


Fig. 4 Delayed-type hypersensitivity (DTH) reaction to *HER2*- and *neu*-protein-expressing cells in rats immunized with *HER2*-ECD. The DTH reaction was assayed 28 days after immunization with *HER2*-ECD. *SKBR-3* lysed *SKBR-3* cells were injected into the left ear, and phosphate-buffered saline into the right ear, of control or immunized animals. *G/DHFR-8* lysed *G/DHFR-8* cells were injected into the left ear, and lysed 3T3 cells into the right ear, of control or immunized animals. [^3H]Thymidine was injected s.c. simultaneously. After 48 h, the ears were removed and prepared for liquid scintillation counting. The figure shows the number of animals per group, and the statistical significance (control versus immunized animals)

In an ELISA to detect anti-(*neu* protein) antibody in rat serum (Fig. 2), 5/14 *HER2*-ECD immune sera gave an absorbance reading more than twice that of the 3 unimmunized control sera, suggesting the presence of cross-reactive antibodies in the immune sera.

Cross-reacting antibodies were also detectable by immunofluorescence. Figure 3 shows the localization of anti-*HER2*-ECD antibodies to the surface of *SKBR-3* cells incubated with serum from *HER2*-ECD-immunized rats (Fig. 3A). No fluorescence was observed with control antibodies or sera. Cell-surface fluorescence could also be seen when *G/DHFR-8* cells were incubated with sera from *HER2*-ECD-immunized animals (Fig. 3B), but not with control sera. As *G/DHFR-8* cells are *neu*-overexpressing 3T3 cells, control 3T3 cells were also tested. Only about 10% of these cells (Fig. 3C) appeared to bind antibody from immune sera, and the fluorescence observed was not localized to the plasma membrane.

In order to measure a possible cellular (DTH) response to *HER2* protein in vivo, the inflammatory response to a *HER2* protein preparation (*SKBR-3* cells) was measured in the ears of rats immunized with *HER2*-ECD (Fig. 4). Lysed *SKBR-3* cells were injected into the left ear, and PBS into the right ear. The ratio of [^3H]thymidine incorporation (left/right ear) was significantly greater ($P < 0.05$) in *HER2*-ECD-immunized animals (geometric mean 1.12, range 0.91–1.37) than in sham-immunized control animals (geometric mean 0.93, range 0.84–1.03). *HER2*-ECD-immunized animals were also tested for a DTH reaction to *neu*.

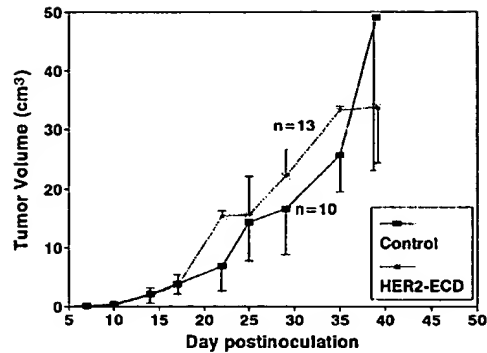


Fig. 5 B104 tumor growth in control and *HER2*-ECD-immunized BDIX rats. B104 cells were injected s.c. into the hind leg. Tumors were measured in three dimensions, and the product taken as the tumor volume

These animals were injected in the left ear with lysed *G/DHFR-8* cells, and in the right ear with lysed control 3T3 cells. A very significant difference ($P < 0.001$) was observed in the [^3H]thymidine incorporation ratios between the control (geometric mean 1.01, range 0.97–1.05) and *HER2*-ECD-immunized animals (geometric mean 1.18, range 0.95–1.47).

As both humoral and cellular cross-reactive responses to *HER2/neu* protein had therefore been demonstrated in *HER2*-ECD-immunized animals, tumors were induced in rats to determine whether *HER2*-ECD immunization could alter the course of tumor growth. Figure 5 shows tumor growth in BDIX rats inoculated s.c. with 2.5×10^6 B104 cells (control curve). Tumors began to appear at about 10 days after inoculation, and 50% mortality was observed within 35–40 days. A second group of rats was immunized with *HER2*-ECD then inoculated 12 days later with B104 tumor cells. No effect on tumor growth was observed in 13 preimmunized animals compared to the 10 control animals that received PBS instead of *HER2*-ECD. Smaller groups of animals were also immunized (a) three times instead of two with *HER2*-ECD, (b) with *HER2*-ECD denatured with dithiothreitol, (c) with *SKBR-3* cells as a source of *HER2* protein, and (d) with lysed B104 cells. None of these immunization protocols showed any effect on the growth of tumors (results not shown).

Discussion

Early work on chemically induced cancers in animals showed that such tumors may express unique tumor-specific transplantation antigens, which could be used as targets for effective immunotherapy [23]. However, more recent experiments have shown that such immunogenic antigens are not generally found on naturally occurring tumors, and that the immunotherapy of these spontaneous tumors may be much more complicated than the earlier work with chemically induced tumors suggested [14]. Most work now focuses on normal cell antigens that show either

a quantitative or qualitative differential expression on tumor cells. In the first case, many tumor-associated differentiation antigens are found on embryonic and tumor cells at relatively high levels, but their expression is reduced in adult cells [13]. Although it is expressed in many fetal tissues, the only adult human cells where the oncogene *HER2* is expressed at higher levels are a subgroup of certain adenocarcinomas, where it is a marker of metastasis and poor prognosis [27]. Although it is not mutated in these cancers, its overexpression appears at least partially to overcome immune tolerance, since both humoral and specific cellular immune responses to this autoantigen have been reported in patients with breast cancers overexpressing *HER2* [5, 6, 17]. More recent studies on cytotoxic T lymphocyte cell lines derived from *HER2*-expressing ovarian tumors support the suggestion that analysis of the private and common *HER2* epitopes may lead to immunotherapeutic uses for this antigen [11]. Qualitative changes in cancer cells, such as oncogene mutation, may also lead to the formation of potentially useful tumor antigens. A human T cell response to mutated *ras* peptides has been demonstrated in follicular thyroid cancer cells [12]. In addition, the fact that a tumor antigen from a spontaneous tumor is not immunogenic in itself does not necessarily preclude its use as an immunotherapeutic target. Van Pel et al. [29] showed that immunogenic tumor cell variants, which failed to grow in the host mouse (tum-), also conferred resistance against challenge with the original tumor cell line (tum+). Only immunization with the tum-variant, but not the original tum+ line provided this resistance.

Although B104 is a chemically induced tumor, it is apparently not very immunogenic and grows quickly in syngeneic BDIX rats [9]. However the expression of *neu* in a mutated form indicates that it may represent a model for breaking tolerance to a tumor-associated autoantigen. Although T cell clonal deletion may be important in the control of an autoimmune response to major antigens, cells reactive to other autoantigens present at lower levels in the body may escape thymic deletion, but be peripherally suppressed in some way (reviewed in [20]). This peripheral tolerance may be broken. Cross-reaction between foreign antigens and autoantigens has been proposed as a possible triggering mechanism in naturally occurring autoimmune disease (reviewed in [19]), and injection of foreign proteins with homology to the target autoantigen has been employed to induce autoimmune disease in animal models. In addition to the example of experimental autoimmune encephalomyelitis mentioned in the introduction, collagen-induced arthritis may be induced in several species by immunization with heterologous type II collagen [30].

Our results showed that such a strategy was effective in the induction of an autoimmune response to the autologous *neu* protein product. The radiometric ear assay results indicated that the DTH response to the *neu* oncoprotein in the *HER2*-immunized animals was at least as strong the response to the immunogen itself. This comparison is even more significant if one takes into account that the animals injected in the left ear with SKBR-3 received only PBS in

the right, there being no suitable control cell preparation for this group of rats. However, only 5/14 animals showed a humoral cross-reaction between *neu* and *HER2* proteins. When these 5 responder animals were analyzed separately, no alteration in tumor growth was observed. This is in contrast to the experience of Drebin [9], who showed that anti-(*neu* protein) antibodies (of mouse origin) were able to reduce B104 tumor growth in BDIX rats.

Although the response to *neu* protein did not appear to alter tumor growth in this model, two points must be taken into account in a general consideration of the future of *HER2/neu* as a potential target for tumor therapy in animal models and in humans. First, B104 tumor growth in BDIX rats is relatively fast, and may not represent an ideal model to detect weak anti-(*neu* protein) immune activity. Secondly, elimination of the whole tumor may not be the only indicator of the potential of *HER2/neu* as a therapeutic target. Anti-(*HER2/neu* protein) responses may induce more subtle phenotypic changes in the tumor. As mentioned above, down-regulation of *HER2* expression with specific antibody leads to a reversal of the malignant phenotype, including a loss of metastatic ability. This may be relevant to the treatment of human tumors in which a more malignant *HER2*-overexpressing subclone may be emerging from the "*HER2*-negative" main tumor mass.

HER2/neu is one of the better characterized oncogenes with potential as a therapeutic target. Further studies in both animal models and in humans must be conducted to realize this potential.

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Thank you.

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Butyrate-inducible and tumor-restricted gene expression by adenovirus vectors

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Efficient introduction of biologically active genes into tumor cells *in vivo* would facilitate cancer gene therapy. In this study, recombinant adenoviruses mediated high-level expression of reporter genes in both human and murine tumor cell lines and freshly resected human solid tumors *in vitro*. When adenovirus vectors were inoculated intratumorally in a living animal, reporter activity was detected only in the tumor nodule and not in other sites, in contrast to intracardiac injections in which expression was found in several organs. Adenovirus-mediated gene expression was further enhanced by butyrate treatment.

Key words: Adenovirus; cancer; therapy; butyrate; luciferase; β -galactosidase.

Many genes have antitumor effects when delivered into tumor cells. The bioactivity of the products encoded by these genes is quite diverse. They may directly inhibit growth,^{1,2} confer sensitivity to drugs,³ or stimulate a host-dependent antitumor immune response by various mechanisms.⁴⁻⁹ It is this last class of genes that is particularly attractive for cancer gene therapy, since they appear to induce immune reactions to both gene-modified tumor cells and their nonmodified counterparts, and as a consequence offer hopes for curing metastatic tumors.

One of the limiting steps in the practical application of these therapies is the difficulty involved in delivering these genes to the relevant tumor cells. Therapeutic genes can be delivered into tumor cells *in vivo* or *in vitro*; however, there are multiple constraints placed by *in vitro* gene transfer. These include (1) the requirement of most gene delivery systems for a dividing target cell population which requires explanted tumors to be adapted to growth in culture, (2) the loss of antigenic characteristics due to outgrowth of subpopulations during *in vitro* culture, and (3) the development of cultured cells is expensive and may cause serious delays of treatment. Introduction of therapeutic genes into tumor cells *in vivo* can bypass many of these problems if sufficient gene expression can be achieved to mount a systemic antitumor immune response or initiate a destructive local reaction. In addition, some genetic approaches, such as tumor eradication mediated by suicide genes,³ exclusively require *in vivo* gene transfer.

Previous attempts have been made to deliver active genes into tumor cells *in vivo* by retrovirus transduction^{3,4} or liposome-mediated transfection.⁴ However, the level and distribution of gene expression, and the susceptibility of different human tumor types have not been determined in a comprehensive manner. Retrovirus vectors deliver genes only into proliferating cells with the appropriate viral receptor.^{10,11} In contrast, adenovirus vectors are capable of transferring and expressing an exogenous gene in dividing, nondividing, or slowly proliferating cells,^{10,11} all of which often coexist in solid tumors. In addition, retrovirus particles are relatively labile while high titers of recombinant adenovirus stocks can be made more easily,^{10,11} thus facilitating the infusion of virus stocks directly into solid tumors in an *in vivo* setting. Adenovirus-mediated transduction is also free of any obvious cytopathic effect. Previous use of live adenovirus vaccines in human populations further demonstrates the safety of this type of vector.^{12,13}

We initiated this study to determine the potential utility of adenovirus vectors for gene therapy of cancer. We delivered a replication-defective human adenovirus serotype 5 vector containing either the firefly luciferase gene (Ad-CMV-*luc*), or a nuclear-targeted *Escherichia coli* β -galactosidase gene (Ad-CMV- β gal) into various human and murine tumors. Both marker genes were under the transcriptional control of the cytomegalovirus (CMV) immediate early enhancer-promoter element. The CMV enhancer is one of the strongest eukaryotic transcriptional signals with little cell type or species specificity.¹⁴ The expression of the luciferase gene provides a quantitative and sensitive measurement for determining the site, degree, and time course of adenovirus-mediated gene expression. The expression of the nuclear-localized β -galactosidase gene in individual cells allows histochemical detection of specific cell types that are transduced by adenovirus vectors among a hetero-

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Table 1. *In vivo* transduction of murine tumor nodules by adenovirus vectors

Mouse	Ad-CMV-luc $\times 10^6$ pfu	Route	Day	BR (mmol/L)	LU/g Tumor $\times 10^4$	LU/g Liver $\times 10^4$	LU/g Spleen $\times 10^4$	LU/g Kidney $\times 10^4$	LU/g Heart $\times 10^4$	LU/g Lung
1	7	IC	3	0	-	6	-	-	430	-
2	7	IC	3	0	-	20	-	2	10	-
3	7	IC	3	0	-	40	3	5	2	-
4	58	IT	3	0	2300	-	-	-	-	-
5	58	IT	3	0	3700	-	-	-	-	-
6	58	IT	3	0	3800	-	-	-	-	-
7	58	IT	3	0	4500	-	-	-	-	-
8	58	IT	3	100	17000	-	-	-	-	-
9	58	IT	3	100	51000	-	-	-	-	-
10	58	IT	3	100	5300	-	-	-	-	-
11	14	IT	1	0	850	ND	ND	ND	ND	ND
12	14	IT	1	0	560	ND	ND	ND	ND	ND
13	14	IT	1	0	250	ND	ND	ND	ND	ND
14	14	IT	4	0	300	-	-	-	-	-
15	14	IT	4	0	270	-	-	-	-	-
16	14	IT	4	0	160	-	-	-	-	-
17	14	IT	4	0	1700	-	-	-	-	-
18	14	IT	4	0	140	-	-	-	-	-
19	14	IT	4	100	9300	-	-	-	-	-
20	14	IT	4	100	770	-	-	-	-	-
21	14	IT	6	0	200	-	-	-	-	-
22	14	IT	6	0	380	-	-	-	-	-
23	14	IT	6	0	320	-	-	-	-	-
24	14	IT	6	0	260	-	-	-	-	-

Note: Inoculations of adenovirus particle suspensions were either intracardiac (IC) in a volume of 0.05 mL, or intratumoral (IT) in a volume of 0.1 mL. Tumors and organs were harvested for luciferase assay 1 to 6 days after virus inoculation. Most tumors weighed 1 to 3 g. pfu, plaque-forming units; -, LU < background²¹; BR, sodium butyrate; 0, no butyrate treatment; 100, 0.2 mL of PBS containing 100 mM of sodium butyrate was infused into a tumor 1 day before the luciferase assay; ND, not determined.

geneous cell population. We found that adenovirus vectors could mediate a high level of gene expression in a variety of different human tumor types. When injected directly into tumors in animals, expression was limited to the tumor deposit. Gene expression could be augmented by butyrate treatment both *in vivo* and *in vitro*. Adenovirus may thus provide a useful vector for expressing exogenous genes in solid tumors.

MATERIALS AND METHODS

Cell cultures

All cell lines and tumor slices used for this study were cultured in RPMI medium 1640 containing 8% fetal bovine serum. Sodium butyrate was prepared as previously described.^{15,16}

Tumor induction

Subcutaneous solid tumors were induced in C57BL/6 mice by inoculating 5×10^5 Lewis lung carcinoma cells into the flank of the animal. One gram of tumor mass contains approximately 10^9 cells. Adenoviruses were usually inoculated into tumors about 10 to 20 days after

tumor induction. The care of animals was in accordance with institutional guidelines.

Luciferase assay

Tumors and organs were homogenized with a Kontes glass tissue grinder in lysis buffer (approximately 0.5 g of tissue in 1 mL of buffer), which was made fresh by mixing buffer A (100 mmol/L potassium phosphate buffer [pH 7.8]/3 mmol/L $MgCl_2$ /1 mmol/L DTT), buffer B (220 mmol/L Tris [pH 7.8]/soybean trypsin inhibitor [1 mg per mL]/aprotinin [0.28 trypsin inhibitor unit per mL]) and 2% NP-40 at a ratio of 14:5:1. After removing cellular debris by centrifugation, up to 50 μ L of tissue extract was used per assay. Luciferase activity was determined with a MGM Optocomp II luminometer by measurement of integrated light emission for 10 seconds in the presence of excess ATP and luciferin.¹⁷ Activity was presented as light units (LU) per gram of tissue or per milligram of soluble protein. Concentration of soluble protein was determined by the BCA (bicinchoninic acid) assay (Pierce Chemical Co, Rockford, Ill). Two $\times 10^6$ LU is equivalent to 1 ng of luciferase as determined by measuring LU produced after exogenously adding purified luciferase enzyme (Sigma Chemical Co, St Louis, Mo) to tumor extracts.

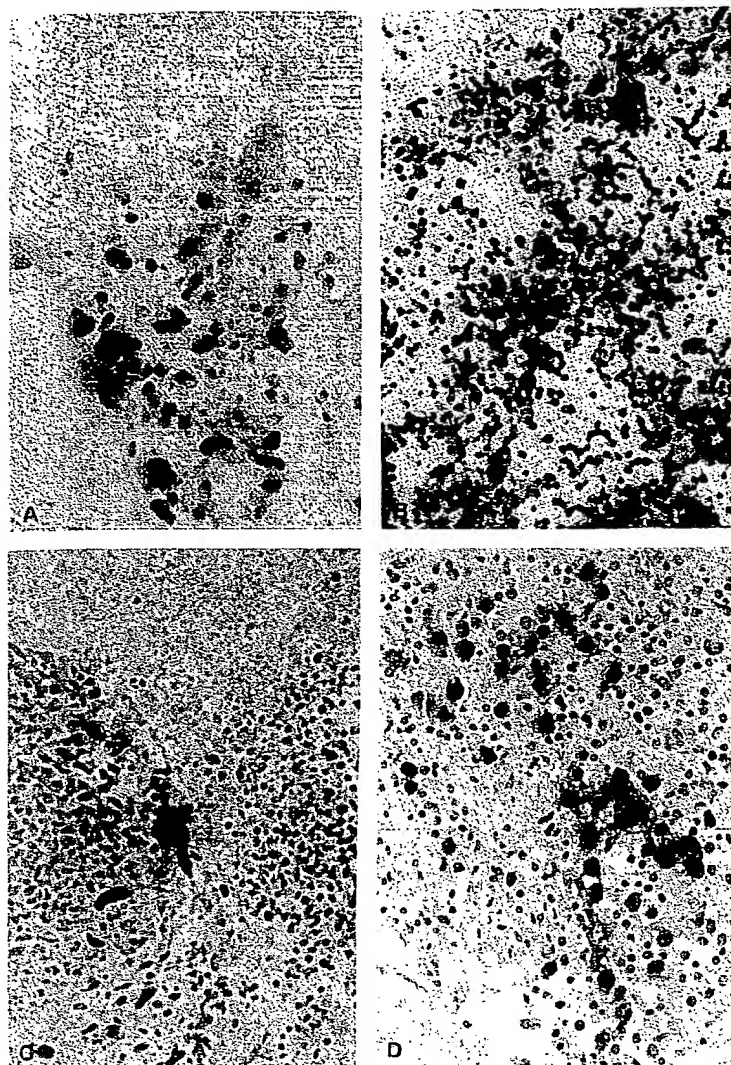


Figure 1. Transfer of the *E. coli* β -galactosidase gene to tumor cells. (A) *In vivo* transduction to solid murine tumors. Tumors recovered from animals 3 days after intratumoral Ad-CMV- β gal inoculation showed expression of the exogenous gene in tumor cells, as seen by nuclear blue staining, which is characteristic of β -galactosidase activity from the *E. coli* β -galactosidase gene coupled with the SV40 nuclear localization signal. Clusters of labelled cells were found near the injection site, while individual labelled cells were visualized elsewhere in the tumor (not shown). (B) High transduction efficiency in a low passage human lung carcinoma cell line (SCC-5) in culture by adenovirus-mediated gene transfer [at a multiplicity of infection of 10 pfu per cell]. (C-D) Histochemical staining of human tumors for β -galactosidase activity. Freshly resected human tumor slices were minced into 2 to 5 mm cubes, incubated in medium containing approximately 10^6 pfu Ad-CMV- β gal for 2 days, and analyzed histochemically with the X-gal stain for detection of β -galactosidase activity. (C) Human lung tumor. (D) Human kidney tumor. (A) and (B) were stained with X-gal only, $\times 200$. (C) and (D) were stained with X-gal, hematoxylin-eosin, $\times 200$. No blue cells were found in any of the untransduced controls.

β -Galactosidase assay

The tumor was minced into 2 to 5 mm cubes, fixed by 4% paraformaldehyde, and stained for β -galactosidase activity by incubation in X-gal staining solution (10 mmol/L $K_4Fe(CN)_6$, 10 mmol/L $K_3Fe(CN)_6$, 1 mmol/L $MgCl_2$, 0.02% NP-40, and 1 mg/mL 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside). Tumors were subsequently embedded in paraffin or frozen in Tissue-Tek O.C.T. compound (Miles Laboratories Inc, Elkhart, Ind) and sectioned. Some sections were counterstained with haematoxylin and eosin.

Adenovirus preparation

The replication-defective human adenovirus serotype 5 derived adenovirus vectors were produced in human 293 cells via homologous recombination between two trans-

duced plasmids containing adenovirus DNA fragments overlapped at the E1a flanking region. A cassette containing marker genes driven by the early enhancer-promoter element of the cytomegalovirus was inserted in place of the E1a deletion.¹⁸ Since the sequences in the E1a region were deleted, the ability of this virus to replicate autonomously in nonpermissive cells was impaired. High titre adenovirus stocks were prepared by ultrafiltration of crude lysates through Amicon Centriprep 100 concentrators.

Gene gun-mediated transfection

Monolayers containing 2×10^6 cells were bombarded in vacuum with 500 μ g of gold microprojectiles (ca 1 μ m in diameter) coated with 4 μ g of plasmid DNA. Expanding helium gas was used as the motive force at a pressure of 1000 psi.^{19,20}

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RESULTS

In vivo transduction of murine tumors

To test the ability of adenovirus vectors to transduce tumors *in vivo*, subcutaneous Lewis lung carcinoma cell tumors were used as targets in syngeneic mice. Systemic administration of Ad-CMV-*luc* by intracardiac injection resulted in reporter gene expression in multiple organs including liver, spleen, kidney, and heart (Table 1). No luciferase activity was detected in the tumor following systemic administration. However, when Ad-CMV-*luc* was injected directly into solid tumors (approximately 1 plaque-forming unit per 20 to 200 tumor cells), luciferase activity was confined to the tumor, most likely due to a high affinity between the virus and the cell. No activity above background (approximately 0.3 pg luciferase per biopsy sample²¹) was detectable in any of the internal organs examined in mice with transduced tumors (Table 1). The level of expression in the tumor was stable for at least 6 days. It has been shown that in some cell types adenovirus-mediated gene expression can last for months.^{10,11} Figure 1A shows that the inoculation of Ad-CMV-*βgal* into a solid tumor *in situ* resulted in groups of labelled tumor cells near the needle track. These data demonstrate that direct inoculation of limiting amounts of recombinant adenovirus vectors into solid tumors can mediate restricted transduction of the tumor with little if any escape to the systemic blood stream and internal organs.

Amplification of expression by butyrate

We wished to determine if adenovirus-mediated gene expression could be further augmented. Figure 2 shows the time course of induction and the effect of treatment with sodium butyrate. When Lewis lung carcinoma cells were transduced by Ad-CMV-*luc* followed by incubation in medium containing 5 mmol/L sodium butyrate for 1 day, luciferase activity was augmented 100-fold. There was no "memory" for butyrate induction as evidenced by a gradual decline of activity after butyrate withdrawal, in contrast to the post-butyrate transcriptional activation observed when a retroviral enhancer-promoter element was induced by transient exposure to butyrate.^{15,16} The butyrate-induced increase of luciferase was unlikely to be due to an enhancement to the stability of luciferase mRNA and/or protein, or a direct effect on the CMV promoter alone, because when a plasmid containing the CMV-luciferase cassette was introduced into a subset of the same population of cells by the gene gun technology¹⁹ as naked DNA, there was only a threefold increase of luciferase activity as a result of butyrate treatment. The increased expression was probably not due to stimulation of virus replication because this adenovirus vector is replication incompetent (E1a deficient). Thus, the induction required the context of a viral infection for reasons that are not understood, but may involve butyrate-induced histone hyperacetylation or other protein modifications (reviewed in ref. 22). To test if this effect could be duplicated *in vivo*, butyrate was infused into solid tumors 2 or 3 days after adenovirus transduction

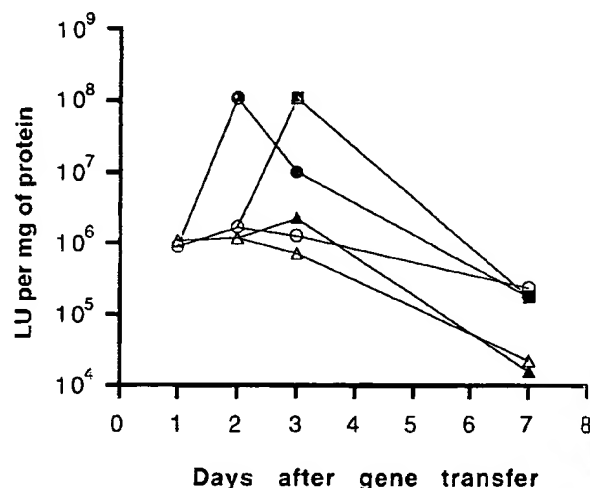


Figure 2. Butyrate induction of adenovirus-mediated gene expression. Lewis lung carcinoma cells cultured were either infected at a multiplicity of infection of 5 pfu per cell by Ad-CMV-*luc*, or bombarded by the gene gun with a plasmid containing the CMV-luciferase transcriptional unit³¹ on day 0. On day 1, genetically modified cells were aliquoted into individual wells in 24-well tissue culture plates. Medium containing 5 mmol/L butyrate was added and withdrawn as described below. Luciferase activity for each well was determined at indicated time points. Each data point represents the mean value from duplicate wells. Open circle, adenovirus-mediated gene expression without butyrate treatment. Solid circle, adenovirus-mediated gene expression treated with 5 mmol/L butyrate between days 1 and 2. Solid square, adenovirus-mediated gene expression treated with 5 mmol/L butyrate between days 2 and 3. Open triangle, gene gun-mediated gene expression without butyrate treatment. Solid triangle, gene gun-mediated gene expression treated with 5 mmol/L butyrate between days 2 and 3. No luciferase activity was detected in control cells without the introduction of the luciferase gene.

and luciferase activity was measured the following day (Table 1). For both measurements, butyrate treatment increased tumor luciferase activity an average of approximately eightfold over untreated counterparts. The highest level was obtained from a butyrate-treated tumor with 5.1×10^8 light units (LU) per gram, which was equivalent to 260 ng of luciferase per gram of tumor. These data suggest that butyrate may be an effective agent for combination therapy with adenovirus vectors.

Transduction of human tumor cells

To extend these findings to human tumors, we transduced an early passage human lung carcinoma cell line (SCC-5) with adenovirus vectors *in vitro*. Figure 1B shows that a single infection with the Ad-CMV-*βgal* virus labelled greater than 80% of these cells in monolayer culture as *β*-galactosidase (+). We then tested freshly resected human solid tumor slices less than 12 hours after removal from patients. Figure 3 shows that adenoviruses could transduce the luciferase gene into all of these tumors. Because the number of viable cells and the number of cells accessible to the virus in each slice

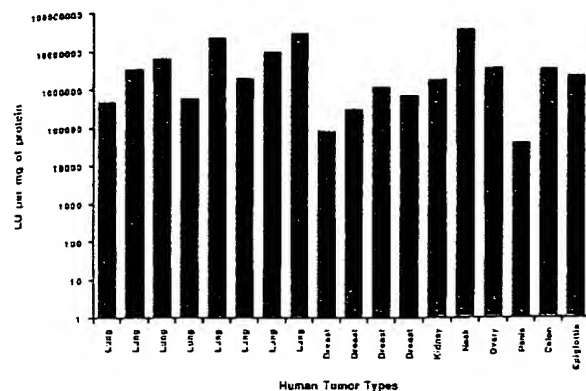


Figure 3. Adenovirus transduction of freshly resected human solid tumors. Fresh human tumor slices less than 12 hours after removal from patients were incubated in medium containing approximately 10^9 pfu of Ad-CMV-*luc* for 2 days. At the end of incubation the tumor was harvested for luciferase assay. No luciferase activity was detectable when mock-transduced slices were assayed as controls. As a cell control, the human lung carcinoma cell line (SCC-5) produced 1.8×10^5 LU/mg of protein after Ad-CMV-*luc* transduction (at a multiplicity of infection of 10 pfu per cell). When a subset of the transduced SCC-5 cells were exposed to butyrate, luciferase activity was augmented to 1.1×10^{10} LU/mg of protein.

were unknown, this tumor-slice assay does not provide quantitative information, although in most cases more luciferase activity was detected when the tumor was treated with butyrate (data not shown). When cultured human lung carcinoma (SCC-5) cells were transduced by Ad-CMV-*luc*, butyrate exposure could also amplify luciferase activity (see Fig 3 legend). Adenovirus vector transduction is greater than 100-fold more effective in SCC-5 than in Lewis lung carcinoma cells (see Fig 2 and legend to Fig 3), although higher butyrate induction was observed in the latter. Both tumor (Figs 1C through 1D) and stromal cells (not shown) in solid human tumors were transduced by Ad-CMV- β gal.

DISCUSSION

This study supports the feasibility of the direct transfer of biologically active genes into tumor cells *in vivo* utilizing recombinant, replication-deficient adenovirus vectors. Several potential scientific and clinical goals could be realized by the application of this approach to cancer treatment. First, adenovirus vectors efficiently transduced every murine and human tumor cell type tested, which makes it likely that this vector will be able to deliver therapeutic genes to most, if not all, human tumors. Second, adenovirus vectors successfully mediated *in vivo* gene delivery to solid tumors after direct inoculation, bypassing the obstacles caused by *in vitro* gene transfer strategies. The tumor-restricted gene expression makes it possible to produce exogenous proteins in tumor deposits with minimal transduction outside the tumor. Third, the expression level mediated by

adenovirus vectors may be higher than other gene transfer techniques (eg, the gene gun [Fig 2], which mediates high transfection efficiencies of DNA into virtually every cell line tested).^{19,20} In addition, butyrate treatment may augment the expression level of genes introduced via adenovirus (Table 1 and Fig 2). Potentially the exogenous gene expression could be further boosted by higher multiplicities of infection or repeated administration of the virus and/or butyrate.

Although the transduced cells were predominantly tumor cells (Fig 1), the normal cells present within the tumor deposit may also be transduced when encountered by the virus. If antiproliferative or suicide genes are transduced, the effect on tumor neovascularity or stromal cells could have an additional antitumor effect. It is also conceivable that a few inoculated viruses may escape the tumor via the bloodstream. It could be argued, therefore, that if immunostimulatory genes are introduced this way, an autoimmune response could be provoked by transduced normal cells. However, previously reported systemic cytotoxic T-cell responses induced after gene-modified tumor cell vaccination have always been tumor specific in spite of the fact that tumor cells have many more "normal" antigens than tumor-specific ones.⁴⁻⁹ Thus, genetic modification of a small number of normal cells may not cause a problem.

We also found that butyrate, a natural lipid component and a fermentation product of carbohydrates, could augment adenovirus-mediated gene expression. The antiproliferative properties of butyrate (reviewed in ref. 22), its potential to induce differentiation of cancer cells,²³⁻²⁸ its low toxicity,^{29,30} and our finding of its ability to activate expression from an adenovirus vector may make it a useful adjunct to adenovirus-mediated cancer gene therapy.

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Thank you..

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Tumor Antigens and Tumor Vaccines: Peptides as Immunogens

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Tumor antigens recognized by human cytotoxic T lymphocytes (CTL) have been identified for multiple types of solid tumors. These include both shared and unique antigens. Unique antigens are those expressed uniquely by one patient's tumor, and shared antigens are those present on tumor cells from many different patients. Many of the shared antigens are derived from tissue-specific differentiation antigens, oncogenes, or a set of antigens expressed only in tumors or in testis. In addition to advances in understanding tumor antigens that stimulate CTL and T-helper cell responses, there have been advances in understanding immunity in general, including the characterization of cytokines, the recognition of the dendritic cell as an optimal antigen-presenting cell (APC), and the characterization of costimulatory molecules as critical components of antigen presentation. Together, these developments have breathed new life into tumor immunology, and they promise to lead to a new generation of peptide- and cell-based tumor vaccines. © 1996 Wiley-Liss, Inc.

KEY WORDS: melanoma, cytotoxic T lymphocytes, neoplasm antigens, peptides/immunology, molecular sequence data, cultured tumor cells, tumor-infiltrating lymphocytes, immunotherapy, immunologic adjuvants

INTRODUCTION

From the early days of W.B. Coley up to the present, surgeons have been leaders in the area of immunotherapy for cancer. Patients who are cured of solid tumors can usually attribute their survival to adequate initial surgical management, but many deaths occur despite appropriate treatment. Existing systemic therapy using cytotoxic chemotherapy has reduced mortality by a significant margin for breast and colon cancers, but melanoma, sarcomas, and many other solid tumors fail to be impacted significantly by adjuvant cytotoxic chemotherapy. Even in breast and colon cancer patients, only a minority of patients derive benefit from this therapy, which is both expensive and morbid. There is, therefore, significant impetus to explore other systemic therapy approaches. Recent advances in tumor immunology offer great promise, especially the new understanding of tumor antigens recognized by T cells.

It has been well demonstrated in animal models that protection against tumor challenge can be obtained by preimmunization with tumor cells [1]. Furthermore, tumor im-

munity can be transferred between syngeneic animals by transfer of lymphoid cells, and adoptive transfer of tumor-reactive cytotoxic T lymphocytes (CTL) has therapeutic efficacy in human cancer patients [2]. Because the principal effector lymphocytes in most model systems are CD8⁺ CTL, these cells have been a major focus of investigation.

In infectious diseases, CTL are responsible for eradication of intracellular pathogens because they recognize antigens comprising peptides generated from intracellular proteins and presented on the cell surface in association with self-major histocompatibility complex (MHC) molecules [3]. Thus, the presence of viral or bacterial proteins in the cytoplasm of host cells is signaled by the presence of peptide fragments recognizable to cytotoxic T cells. Similarly, tumor-specific CTL populations generated in vitro from the lymphocytes of patients with solid tumors recognize tumor-derived proteins presented by MHC molecules.

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PEPTIDE EPITOPES FOR TUMOR-REACTIVE CYTOTOXIC LYMPHOCYTES

In 1991, the first tumor antigen recognized by T cells in a murine mastocytoma was reported [4,5]. Since that time, there has been rapid development in the field of tumor immunology and, specifically, in the area of antigen identification. Tumor antigens recognized by human CTL have been identified for multiple types of solid tumors. These include both shared and unique antigens; unique antigens are those expressed uniquely by one patient's tumor, and shared antigens are those present on tumor cells from many different patients. For melanoma [6-8], sarcomas [9], ovarian cancer [10-13], and adenocarcinomas of the breast [14,15] and colon [16,17], there has been convincing evidence, in one or more studies, of T cells reactive against antigens that are shared by multiple tumors from different individuals.

Significant effort has been placed on identifying the specific peptide epitopes responsible for shared tumor antigens because they offer the potential for use as immunogens in specific active immunotherapy (tumor vaccines) for the prevention and treatment of cancer. In addition, there have been hopes that characterization of all tumor antigens (shared or unique) may open a window on the process of malignant cellular transformation. Further, the great paradox at the heart of tumor immunology is that patients who do die of cancer have circulating lymphocytes that are capable of recognizing and destroying those same cancer cells that are growing in vivo. Greater understanding of the specific epitopes for cytotoxic T cells, whether shared or unique, may provide a tool to permit understanding of the failure of the host immune response in patients experiencing cancer progression.

Using a combination of approaches, a number of peptide epitopes for human tumor-reactive CTL have now been identified. The proteins from which these peptides have been derived can be grouped into five categories (Table I). Prior to identification of tumor-associated CTL epitopes, oncogenes were considered a likely source of those epitopes. Although oncogene-derived epitopes have been defined, the majority described to date are derived from nonmutated proteins other than known oncogenes. Most of the defined epitopes have

been derived from studies of human melanoma, and shared melanoma epitopes are derived predominantly from two categories of normal proteins: the melanocytic tissue differentiation antigens gp100/Pmel-17, tyrosinase, MART-1/Melan-A, and gp75/trp-1 and the MAGE/BAGE/GAGE gene families. A few peptides resulting from mutations in gene sequences of the tumor cells have been described. The number in this category is likely to increase as more attention is focused on antigens unique to individual tumors.

The peptides that comprise epitopes for CTL are presented by Class I MHC molecules and are usually 9 amino acids in length. These 9-mers account for most of the described CTL epitopes, but 8-mers, 10-mers, and—less commonly—longer peptides have also been described [see ref. 50]. A summary of described peptide epitopes for human tumor-specific CTL is listed in Table II [18-49]. These peptides have been identified by a number of different methods. Genetic approaches pioneered by Boon use DNA libraries of melanoma cells to identify novel genes from which CTL epitopes are derived. Alternatively, mass spectrometric evaluation of MHC-associated peptides has permitted direct sequencing of the naturally processed peptides. The other general approach has been to identify a particular protein or gene as a possible source of peptides and then to evaluate that hypothesis either after inducing its expression into a nontumor cell or by synthesizing peptides encoded by the gene, based on known MHC-binding motifs. All of these approaches have been used successfully. Ideally, the different methods are corroborative. An example is the series of reports on gp100/Pmel-17-derived peptides. Bakker et al. [25] hypothesized that this protein may be a source of CTL epitopes, based on correlations between reactivity of the anti-gp100 monoclonal antibody HMB-45 and sensitivity to CTL recognition of multiple tumors. They went on to prove that hypothesis. Independently, a gp100/Pmel-17-derived peptide YLEPGPVTA was identified directly on the HLA-A2 molecules of melanoma cells and was found to be capable of reconstituting a CTL epitope [22]. Subsequently, several additional gp100/Pmel-17-derived peptides have been identified [23,24].

It has generally been true that epitopes predicted from a defined gene sequence correspond to the actual processed

TABLE I. Proteins Encoding Peptide Epitopes for Human Tumor-Specific CTL

Category of antigenic protein	Example(s) for which specific epitopes have been described or postulated
Oncogenes (mutated or overexpressed)	HER-2/neu, mutated ras
Mutated cellular proteins other than known oncogenes	beta-catenin
Nonmutated tissue-specific proteins	Tyrosinase, gp100/Pmel-17, MART-1/Melan-A, gp75/trp-1
Nonmutated proteins expressed in cancer cells and testis	MAGE-1, MAGE-3, BAGE, GAGE-1 and -2
Viral proteins	E6, E7 of HPV

HPV = human papilloma virus

TABLE II. Peptide Epitopes for Human Tumor-Specific CTL

Protein	MHC restriction	Peptide sequence	Tumor type	Reference
Tyrosinase	A1	Not yet identified	Melanoma	Kittleson (unpublished observations)
Tyrosinase	A2	YMDGTMSQV	Melanoma	Skipper et al. [18]
Tyrosinase	A2	MLLAYLYCL	Melanoma	Wolfel et al. [19]
Tyrosinase	A24	AFLPWHRLF	Melanoma	Wolfel et al. [19]
		AFLPWHRLF		Kang et al. [20]
Tyrosinase	B44	SEIWRDIDF	Melanoma	Brichard et al. [21]
gp100/Pmel-17	A2	YLEPGPVTA	Melanoma	Cox et al. [22]
gp100/Pmel-17	A2	KTWGQYWQV	Melanoma	Bakker et al. [23]
				Kawakami et al. [24]
gp100/Pmel-17	A2	ITDQVPFSV	Melanoma	Kawakami et al. [24]
gp100/Pmel-17	A2	VLYRYGSFSV	Melanoma	Kawakami et al. [24]
gp100/Pmel-17	A2	LLDGATLRL	Melanoma	Bakker et al. [25]
				Kawakami et al. [26]
gp100/Pmel-17	A3	ALLAVGATK	Melanoma	Skipper et al. [27]
MART-1/Melan-A	A2	AAGIGILT	Melanoma	Coulie et al. [28]
				Kawakami et al. [29]
MART-1/Melan-A	A2	ILTVILGVL	Melanoma	Castelli et al. [30]
gp75/tp-1	A31	—	Melanoma	Wang et al. [31]
MAGE-1	A1	EADPTGHSY	Melanoma, other tumors ¹	Traversari et al. [32]
MAGE-1	Cw*1601	SAYGEPRKL	Melanoma, other tumors ¹	van der Bruggen et al. [33]
MAGE-3	A1	EVDPIGHLY	Melanoma, other tumors ²	Celis et al. [34]
				Gaugler et al. [35]
MAGE-3	A2	FLWGPRALV	Melanoma, other tumors ²	van der Bruggen et al. [36]
BAGE	Cw*1601	AARAVFLAL	Melanoma, other tumors ³	Boel et al. [37]
GAGE-1,2	Cw6	YRPRPRRY	Melanoma, other tumors ⁴	Van den Eynde et al. [38]
HER-2/neu	A2	KIFGSLAFL	Ovarian cancer	Fisk et al. [39]
		VMAGVGSPYV		Lustgarten and Sherman [40]
HER-2/neu	A2	HISAVVGIL	Ovarian cancer, non-small cell lung cancer	Yoshino et al. [41]
				Linehan et al. [42]
				Peoples et al. [43]
				Yoshino et al. [44]
CEA	A2	YLSGANLNL	Colon cancer	Tsang et al. [17]
p15	A24	(E)AYGLDFYIL	Melanoma and normal tissues	Robbins et al. [45]
43 kD protein	A2	QDLTMKYQIF	Melanoma	Morioka et al. [46]
MUM-1 gene product mutated across intron/exon junction	B*4402	EEKLIVVLF ⁵	Melanoma	Coulie et al. [47]
Mutated beta-catenin	A24	SYLDSGIHF ⁶	Melanoma	Rosenberg [48]

¹MAGE-1: expressed in melanoma (36%), bladder (19%), breast (18%), head and neck (25%), and non-small cell lung cancers (34%), sarcoma (11%), and prostate cancer (15%) [49].

²MAGE-3: expressed in melanoma (65%), bladder (34%), breast (11%), head and neck (48%), and non-small cell lung cancers (31%), sarcoma (11%), and prostate cancer (15%) [49].

³BAGE: expressed in melanoma (22%), bladder (15%), breast (10%), head and neck (<10%), and non-small cell lung cancers (<10%) [49].

⁴GAGE-1, -2: expressed in melanoma (24%), bladder (12%), breast (9%), head and neck (19%), and non-small cell lung cancers (19%), sarcoma (25%), and prostate cancer (10%) [49].

⁵Isoleucine (I) at position 5 is the result of mutation. The wild-type sequence is EEKLSVVLFF.

⁶Phenylalanine (F) at position 9 is the result of mutation. The wild-type sequence is SYLDSGIHFS.

peptide epitope recognized by CTL. However, antigen processing may occur after posttranslational modifications. In one case, N-linked glycosylation and subsequent deglycosylation result in conversion of an asparagine residue to aspartic acid in the tyrosinase protein, such that a tyrosinase-derived peptide epitope is YMDGTMSQV instead of YMNGTMSQV [18]. The biologic effect of this change is so significant that the naturally processed peptide YMDGTMSQV reconstitutes the epitope for

tyrosinase-specific CTL at a concentration several orders of magnitude lower than that required for the peptide predicted from the gene sequence. Thus, it is valuable to have corroborative data on epitope identification from more than one methodology.

PEPTIDE EPITOPES FOR HELPER T CELLS

The cytotoxic T-cell response to cancer generated in vitro depends on supplementation of the culture media

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with recombinant interleukin (IL)-2, which replaces IL-2 generated in vivo by helper T cells. The in vivo immune response to tumor cells presumably depends on recognition of epitopes by helper T cells (classically CD4+) as well as recognition of epitopes by cytotoxic T cells [51]. The former recognize peptides presented by Class II MHC molecules. These peptides differ from those presented by class I MHC molecules in that they are much longer, usually ranging from 12 to 19 amino acids in length [52]. These have been less well studied and are more difficult to sequence because of their length, however, it is reasonable to predict that the tumor-associated molecules that give rise to antigenic peptides presented on Class I MHC molecules (A, B, C) will also give rise to antigenic peptides presented on Class II MHC molecules (DR, DP, DQ). Efforts to identify tumor-specific Class II-restricted peptides are underway, and tyrosinase has been shown to encode at least one epitope recognized by CD4+ (helper) T cells, and presented by HLA-DR molecules [53]. Class II MHC-restricted helper T-cell responses have been identified for several cancers. Limited data known about these responses are listed in Table III.

PEPTIDE-BASED TUMOR VACCINES

The identification of peptide epitopes for tumor-specific CTL has made possible the development of peptide-based tumor vaccines for human use. Many such vaccines are now in phase I clinical trials, but there is no consensus about how best to immunize against cancer with peptides. In part, this is due to incomplete understanding of the nature of the host:tumor relationship. Tumor-infiltrating lymphocytes (TIL) cultured in the presence of IL-2 develop tumor-specific reactivity in some cases, and this is offered as evidence of tumor-specific immune responses occurring in vivo [62]. On the other hand, cytokines produced by tumor cells and TIL in situ are often those associated with immunosuppression (transforming growth factor [TGF]-beta, IL-10) rather than those associated with immune activation (IL-2, gamma-interferon [IFN], tumor necrosis

factor [TNF]-alpha) [63-68]. Tumor-induced immune suppression has been reported in some tumor-draining nodes [69-72] and may explain the failure of TIL to eradicate tumor deposits. A possible explanation is anergy due to absence of the costimulatory molecules B7-1 and B7-2 on most tumor cells [73]. Thus, T-cell interactions with antigens presented on the melanoma cells may result in anergy rather than immunity [74]. To overcome anergy and to induce immunity, specific stimulation in the correct immunologic milieu is critical.

It appears that classical concepts of tolerance based on self and nonself are incomplete and that immunity can be generated against antigens for which tolerance is preexisting [75]. A goal of effective immunization is the presentation of antigenic peptides on the MHC molecules of dendritic cells or other antigen-presenting cells (APCs) and the expression of appropriate cytokines locally at the immunization site and at draining nodes, ultimately resulting in creation of systemic immunity.

Murine studies have shown that dendritic cells pulsed with immunogenic peptides induce protective immunity against tumors that express those peptides. More importantly, this approach induces sufficient immunity that established tumors can be destroyed in multiple different murine tumor systems [76-78]. In humans, this approach will require harvesting dendritic cells from peripheral blood, then adoptive transfer of the dendritic cells after pulsing them with immunogenic peptides. This is a very promising approach, and several centers are bringing this into human clinical trials, especially for melanoma patients.

Other approaches include direct peptide immunization or immunization with peptide plus adjuvant. It has been expected that an adjuvant will be needed; however, a preliminary report of a phase I trial of naked peptide without adjuvant (MAGE-3 peptide) found that one half of the six evaluable patients experienced partial responses with this simple approach [79]. Augmentation of these responses may be expected if an effective adjuvant is added. A goal,

TABLE III. Class II MHC-Restricted T-Cell Responses to Human Solid Tumors

Cancer type	Protein source	Restricting MHC class II allele	Reference
Melanoma	Tyrosinase	DR	Topalian et al. [53]
Melanoma	Not reported	DR2	Chen and Hersey [54]
Melanoma	Not reported	DR7	Radrizzani et al. [55]
Melanoma	Not reported	DR15	Takahashi et al. [56]
Breast cancer	HER-2/neu ¹	Not reported	Disis et al. [57]
Colon cancer	CTAA 28A32-32K (annexin-related protein)	Not reported	Ransom et al. [58]
Ovarian cancer	Not reported	Not reported	Ioannides and Freedman [59]
Pancreatic cancer	Mutant ras	Not reported	Qin et al. [60]
Renal cell cancer	Not reported	Not reported	Weidmann et al. [61]

¹HER-2/neu peptides inducing CD4 responses include HLDMLRHLYQ GCQVV (amino acid residues 42-56) and SRLLGICLTS TVQLV (amino acid residues 783-797).

therefore, is the identification of optimal adjuvants for stimulating CTL responses to purified peptide. Although Bacillus Calmette-Guérin vaccine (BCG) does induce CTL responses, the local reaction is quite morbid, and serious reactions can occur. Alum is not effective for CTL responses, despite its use as an adjuvant for generating antibody responses. Adjuvants currently being studied are various preparations that resemble incomplete Freund's adjuvant; bacterial cell wall-derived preparations that have been modified to reduce toxicity; and other experimental adjuvants of other classes. Each has been used to some extent in humans, with variably positive results. Many of the ongoing clinical trials are using one or more of these adjuvants. To be successful, an adjuvant probably must be capable of stimulating a local inflammatory response with release of appropriate cytokines for stimulation of dendritic cells and other APCs and for support of T-cell responses.

Since granulocyte-macrophage colony-stimulating factor (GM-CSF) is critical for dendritic cell activation, it has attracted attention as a cytokine adjuvant [80]. Similarly, IL-12 is being studied as a vaccine adjuvant because of its role in directing Th1 responses and, thus, CTL responses [81]. Therefore, one approach is to vaccinate with peptide plus systemic or local administration of IL-12 or GM-CSF. Local administration may be accomplished by gene therapy methods.

In addition to direct administration of peptides, other methods for inducing expression of known tumor antigens involve introducing minigenes encoding antigenic peptides directly into normal cells either as naked DNA (gene-gun or other approaches) or via a viral vector. These approaches can also be modified by coadministration of genes encoding costimulatory molecules or cytokines. This assumes that the antigenic peptides will be presented, via endogenous processing, on cells expressing the appropriate costimulatory molecules. Vaccinia-based vaccines are attractive because of the extensive safety data available from the smallpox vaccination era, because of the wide host range of vaccinia viruses, and because of the adjuvant effect of vaccinia infection. However, an additional concern is that repeat vaccination with vaccinia may result in such rapid eradication of the virally infected cells that the tumor antigens being expressed may not be effectively presented to the immune effector cells.

A clinical trial of carcinoembryonic antigen (CEA)-expressing vaccinia vaccination for colorectal cancer has been initiated despite those concerns, as has a vaccinia approach using the human papilloma virus (HPV)-derived antigens E6 and E7 for cervical cancer. The CEA-vaccinia trial has resulted in some cell-mediated immune responses against at least one CEA-derived peptide, and rejection of vaccinia itself has not been as much of a problem as feared [17]. Despite these promising early results with vaccinia-based strategies, other viral vectors are being developed for vaccine use, often with modification to reduce immuno-

genicity or toxicity. Viral vectors under investigation include poxviruses, herpes viruses, and retroviruses, among others.

Another provocative approach to vaccination with defined antigens is to use *Listeria monocytogenes* to introduce DNA encoding the antigenic proteins. *Listeria* is a human pathogen which rarely produces symptoms but which infects cells as an intracellular pathogen. Proteins encoded by its genome are secreted into the cytoplasm and into lysosomes, resulting in processing and presentation on Class I and Class II MHC molecules by their respective pathways. Thus, this may well be another promising approach for gene-based peptide/antigen vaccination.

It is expected that the next few years will be marked by many clinical trials of peptide-based tumor vaccines for melanoma and for other tumors. The result should be a relative consensus on optimal ways to induce peptide-specific immune responses by immunization. There will also be improved understanding of the host-tumor relationship. As effective methods of vaccination are developed, it will become increasingly important to understand how some tumor cells evade immune recognition. Several methods for immunologic escape have been described, including down-regulation of MHC molecule expression [82-85], defects in antigen processing [86], and loss of expression of immunogenic proteins [87]. Our experience with a panel of human melanoma lines that evade immune recognition is that they all escape immune recognition by failing to express any of three immunogenic melanocytic differentiation proteins and, presumably, several other proteins not yet identified (manuscript in preparation). Whether this is due to immune selection in vivo or whether it is due to major phenotypic differences in melanoma subtypes that are not yet well elucidated, this phenomenon will affect immunotherapy significantly. Melanomas and most other tumors are heterogeneous in their expression of numerous antigens, and clonal selection is, presumably, part of the process of metastasis and progression. Although over 95% of primary melanomas are pigmented and also express gp100/Pmel-17, for example, metastatic melanoma is more commonly amelanotic than the primary lesions, and expression of the antigenic melanoma protein gp100/Pmel-17 is decreased on metastatic vs. primary lesions [88].

A subset of melanomas may not express any of the melanocytic differentiation antigens gp100/Pmel-17, gp75/trp-1, MART-1/Melan-A, or tyrosinase (manuscript in preparation). Except for MAGE-3, only a minority of melanomas express each of the antigens of the MAGE, BAGE, and GAGE gene families [49], and it is not yet clear what percentage of melanomas fail to express all of these antigens. However, even tumors that escape recognition by CTL that recognize multiple shared antigens may express antigens recognized by autologous CTL. Thus, there is reason to be optimistic that tumors that escape immune recognition by deletion of specific antigens can be

eradicated by immunotherapy directed at other antigens. There are already two examples of patients whose melanomas were dramatically decreased by immunotherapy, only to recur later, at which time deletion of MHC molecules or of target antigen expression was noted. However, CTL could still be generated *in vitro* against other antigens on the existing tumor [45,89]. In one of these cases, a second round of immunotherapy directed at a newly defined antigen was successful [45].

In summary, the antigenic profile of a human tumor is complex, consisting of antigenic peptides derived from many different classes of protein. Many of the shared antigens have been identified, a substantial subset of which are tissue-specific differentiation antigens, oncogenes, or a set of antigens expressed only in tumors or in testis. The ideal vaccine for cancer must take into account its heterogeneity and its ability to modulate expression of tumor antigens and other proteins important for immune effect. Thus, an optimal vaccine will likely incorporate a large panel of immunogenic peptides or the proteins from which they are derived. The fact that unique antigens also exist and may be critical to immune responses may require that antigens unique to the autologous tumor be incorporated in the vaccine cocktail. Thus, custom vaccines could be generated by identifying specific peptides for each tumor; however, this is likely to be prohibitively difficult to do on a case-by-case basis.

Alternatively, the autologous tumor cells themselves express all of the antigens of interest for a given patient. The concept of vaccinating with autologous tumor cells is not a new one, but realization of the importance of costimulatory molecules may direct the next generation of cell-based tumor vaccines toward transforming the tumor cells into APCs effectively presenting antigens plus costimulatory molecules; alternatively, expression of dendritic cell-inducing cytokines such as GM-CSF may induce more effective immunity, presumably by permitting dendritic cells to present melanoma cell-derived proteins in an effective manner. This approach is currently being investigated in several centers.

There have been many advances in understanding tumor antigens that stimulate CTL and T-helper cell responses. Furthermore, there have been advances in understanding immunity in general, including the characterization of cytokines such as GM-CSF and IL-12, the recognition of the dendritic cell as an optimal APC, and the characterization of costimulatory molecules as critical components of antigen presentation. Together, these developments have breathed new life into tumor immunology and they promise to lead to a new generation of peptide- and cell-based tumor vaccines. Key considerations are to ensure that the new enthusiasm remains governed by a keen sense of caution, founded in ethical medical practices and in sound immunologic principles. The result is certain to be a much better understanding of the role of the immune system in controlling solid tumors in humans.

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Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines.

Fisk B, Blevins TL, Wharton JT, Ioannides CG.

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Department of Gynecologic Oncology, University of Texas, M.D. Anderson Cancer Center, Houston 77030, USA.

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Synthetic peptide analogues of sequences in the HER-2 protooncogene (HER-2) were selected based on the presence of HLA-A2.1 anchor motifs to identify the epitopes on HER-2 recognized by ovarian tumor-reactive CTL. 19 synthetic peptides were evaluated for recognition by four HLA-A2 ovarian-specific cytotoxic T lymphocyte (CTL) lines obtained from leukocytes associated with ovarian tumors. The nonapeptide E75 (HER-2, 369-377:KIFGSLAFL) was efficient in sensitizing T2 cells for lysis by all four CTL lines. This peptide was specifically recognized by cloned CD8+ CTL isolated from one of the ovarian-specific CTL lines. E75-pulsed T2 cells inhibited lysis by the same CTL clone of both an HLA-A2+ HER-2high ovarian tumor and a HER-2high cloned ovarian tumor line transfected with HLA-A2, suggesting that this or a structurally similar epitope may be specifically recognized by these CTL on ovarian tumors. Several other HER-2 peptides were recognized preferentially by one or two CTL lines, suggesting that both common and private HER-2 epitopes may be immunogenic in patients with ovarian tumors. Since HER-2 is a self-antigen, these peptides may be useful for understanding mechanisms of tumor recognition by T cells, immunological tolerance to tumor, and structural characterization of tumor antigens.

PMID: 7539040 [PubMed - indexed for MEDLINE]

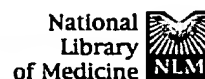
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Immunogenicity of GM-CSF products in cancer patients following immunostimulatory therapy with GM-CSF.

Wadhwa M, Mellstedt H, Small E, Thorpe R.

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Division of Immunobiology, National Institute for Biological Standards and Control, Potters Bar, Herts, UK. mwadhwa@nibsc.ac.uk

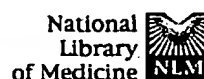
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There is a high risk of developing neutralising and non-neutralising antibodies when GM-CSF is used as an immunomodulatory agent in non-immunocompromised patients. The presence of neutralising antibodies may seriously hamper the clinical response of the patients. This must be taken into account when designing protocols if the biological activity of the exogenously administered GM-CSF is not to be impaired and the endogenous production of GM-CSF is not to be inactivated. Assessment of production of neutralising antibodies during cytokine therapy is important for predicting the clinical response to progressive therapy. Use of validated assays is imperative for evaluation of antibodies generated following therapy with a particular protein.

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Humoral and cellular responses raised against the human HER2 oncoprotein are cross-reactive with the homologous product of the new proto-oncogene, but do not protect rats against B104 tumors expressing mutated neu.

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Taylor P, Gerder M, Moros Z, Feldmann M.

Center for Experimental Medicine, Venezuelan Institute for Scientific Research, IVIC, Caracas, Venezuela. ptaylor@medicina.ivic.ve

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The neu proto-oncogene encodes a plasma membrane protein belonging to the epidermal growth factor receptor family. The cell line B104, derived from BDIX rat neuroblastoma, carries a point mutation in neu, and forms a tumor when injected into these rats. The human homologue of the neu oncogene (here called HER2) is overexpressed in certain types of cancer. Rats were immunized with HER2 protein (HER2) to investigate a possible cross-reaction between the homologous proteins which could protect them against subsequent inoculation with B104. Specific antibody in the serum was measured by cell-based enzyme-linked immunoabsorbent assay and fluorescence immunocytochemistry, and delayed-type hypersensitivity by an ear assay. Sera from animals immunized with the HER2 extracellular domain (HER2-ECD) reacted with both HER2- and neu-expressing cells. In the ear assay, a significant cellular response to both HER-ECD ($P < 0.05$) and neu protein ($P < 0.001$) was observed in HER2-ECD-immunized rats. However, the growth of B104 tumors in rats was not affected by preimmunization with HER2-ECD. The results indicate that an autoreactive immune response to neu was induced by immunization with HER2-ECD, but was too weak to affect the growth of the neu-bearing tumor.

PMID: 8640846 [PubMed - indexed for MEDLINE]

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HER2/neu-derived peptides are shared antigens among human non-small cell lung cancer and ovarian cancer.

Yoshino I, Goedegebuure PS, Peoples GE, Parikh AS, DiMaio JM, Lyerly HK, Gazdar AF, Eberlein TJ.

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Division of Surgical Oncology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115.

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Previously, we have reported a correlation between the expression of HER2/neu and sensitivity to HLA-A2-restricted cytotoxic T-cells (CTL) in ovarian cancer. To investigate the role of HER2/neu in human non-small cell lung cancer (NSCLC), we established autologous tumor-specific CTL from tumor-infiltrating lymphocytes of HLA-A2+ HER2/neu+ NSCLC patients. These CTL lines specifically recognized HLA-A2+ HER2/neu+ autologous and allogeneic NSCLC cell lines as well as HLA-A2+ HER2/neu+ heterologous ovarian cancer cell lines. Furthermore, these CTL recognized an overexpressed, HER2/neu-derived peptide. From these results, we conclude that HLA-A2 serves as a restriction element in NSCLC. More importantly, at least one HER2/neu-derived peptide is a tumor-associated antigen in NSCLC and ovarian cancer.

PMID: 7912166 [PubMed - indexed for MEDLINE]

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Granulocyte-macrophage colony-stimulating factor: an effective adjuvant for protein and peptide-based vaccines.

Disis ML, Bernhard H, Shiota FM, Hand SL, Gralow JR, Huseby ES, Gillis S, Cheever MA.

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Department of Medicine, University of Washington, Seattle 98195-6227, USA.

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The current studies evaluate granulocyte-macrophage colony-stimulating factor (GM-CSF) as a vaccine adjuvant. An important issue for developing vaccine therapy for human malignancy is identifying adjuvants that can elicit T-cell responses to proteins and peptides derived from "self" tumor antigens. GM-CSF, in vitro, stimulates the growth of antigen-presenting cells such as dendritic cells and macrophages. Initial experiments examined whether GM-CSF injected into the skin of rats could affect the number or character of antigen presenting cells, measured as class II major histocompatibility complex expressing cells, in lymph nodes draining the injection site. Intradermal (id) inoculation of GM-CSF every 24 hours for a total of five inoculations resulted in an increase of class II+ fluorescing cells that peaked at the fourth inoculation. Subcutaneous (sq) inoculation resulted in an increase of class II+ fluorescing cells that peaked following the second inoculation, then decreased over time. Using this schema for "conditioning" the inoculation site, GM-CSF was administered id or sq for five injections and a foreign antigen, tetanus toxoid (tt), was given at the beginning or the end of the immunization cycle. Id immunization was more effective than sq at eliciting tt specific immunity. In addition, GM-CSF id, administered as a single dose with antigen, compared favorably with complete Freund's adjuvant (CFA) and alum in eliciting tt specific antibody and cellular immunity. We have shown that immunity to rat neu (c-erbB-2) protein, an oncogenic self protein, can be generated in rats by immunization with peptides derived from the normal rat neu sequence plus CFA. The current study demonstrates that rat neu peptides inoculated with GM-CSF could elicit a strong delayed type hypersensitivity reaction (DTH) response, whereas peptides alone were non-immunogenic. GM-CSF was as effective as CFA in generating rat neu specific DTH responses after immunization with a neu peptide based vaccine. Soluble GM-CSF is a potent adjuvant for the generation of immune responses to foreign proteins as well as peptides derived from a self tumor antigen.

PMID: 8704175 [PubMed - indexed for MEDLINE]



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Dendritic cells as antigen presenting cells in vivo.

Inaba K, Metlay JP, Crowley MT, Witmer-Pack M, Steinman RM.

Department of Zoology, Faculty of Science, Kyoto University, Japan.

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The biology of antigen presenting cells (APC) traditionally is studied in tissue culture systems using T cells that have been expanded beforehand by stimulation with antigen. Here we consider the distinctive roles of dendritic cells for sensitizing or priming T cells both in vitro and in vivo. Several functions of dendritic cells have been identified in tissue culture that are pertinent to T cell sensitization. These include the ability to a) capture and retain foreign antigens in an immunogenic form, b) bind antigen-specific resting lymphocytes, and c) activate T cells to produce lymphokines and undergo long term clonal growth. Dendritic cells have several properties in vivo that also would contribute to APC function. These are a) their widespread tissue distribution permitting access to antigens in most organs, b) the capacity to home via the blood stream and afferent lymph to the T-dependent areas of spleen and lymph node, and c) the ability to capture antigen in antigen-pulsed animals. Dendritic cells bearing antigen have been administered in situ to initiate responses like contact sensitivity, graft rejection, and antibody formation. A most striking recent example is that, when dendritic cells are pulsed with protein antigens in vitro and administered to immunologically naive mice, there is direct priming of antigen-specific T cells that are restricted to the MHC of the injected APC.

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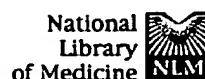
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HER2 Dendritic Cell Vaccines.

Morse MA, Clay TM, Colling K, Hobeika A, Grabstein K, Cheever MA, Lyerly HK.

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HER2/neu, a tumor antigen overexpressed by a third of breast cancers, is a potential target for vaccine therapies. A particularly potent immunization strategy to induce T-cell responses against tumor antigens is to use dendritic cells (DCs) loaded with the tumor antigen. We performed two small studies to test the safety, feasibility, and immunologic and clinical responses to immunizations with in vitro-generated DCs loaded with either a human leukocyte antigen A2-restricted peptide fragment of the extracellular domain of the tumor antigen HER2 (E75) or a HER2 intracellular domain (ICD) protein in patients with high-risk resected breast cancer or metastatic cancers expressing HER2. There were no toxicities due to the immunizations in any of the patients. In the study of DCs loaded with the E75 peptide, 1 of 6 patients with metastatic HER2-expressing malignancies who completed all immunizations had stable disease for 6 months; the remainder of the patients had progressive disease. Delayed-type hypersensitivity (DTH) reactivity (2-3 mm of induration) at E75-loaded DC injection sites was observed in 2 of 5 patients evaluated but was similar at the unloaded DC injection sites. In 2 patients, the DTH sites underwent biopsy and a perivascular infiltrate of CD4 and CD8 cells was demonstrated, which was greater in the E75-loaded DC injection sites than in the unloaded DC sites. In the pilot study of ICD-loaded DC in patients with high-risk resected breast cancer, all 3 patients enrolled had no evidence of recurrence at a follow-up of up to 2.5 years. Intracellular domain-specific T-cell responses were detected directly from the peripheral blood by enzyme-linked immunospot and proliferation assay in 2 patients. We conclude that it is feasible and safe to generate and administer HER2-loaded DCs to patients with advanced HER2/neu-expressing malignancies and high-risk breast cancer. The magnitude of the immune responses generated is fairly modest, and more potent DC loading and maturation strategies will be necessary to optimize these vaccines.

PMID: 12620155 [PubMed - in process]



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☐ 1: Eur J Clin Microbiol Infect Dis. 1994;13 Suppl 2:S47-53.

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The uses of GM-CSF as an immunomodulator and vaccine adjuvant are reviewed. GM-CSF has a variety of effects on immune responses: it induces class II major histocompatibility complex antigen expression on the surface of macrophages; it enhances dendritic cell maturation and migration; it results in a localized inflammation at the injection site; and it has marked effects on maturation of haematopoietic progenitor cells in the bone marrow. Animal and human studies suggest that administration of GM-CSF can increase antibody titres to foreign antigens. Monkeys injected with human interleukin (IL)-3 plus GM-CSF, at a different injection site, developed peak antibody titres which were 8- to 30-fold higher than those in monkeys injected with IL-3 alone. In a study of ovarian cancer patients receiving GM-CSF to prevent chemotherapy-induced neutropenia, two patients who had demonstrated a low titre of antithyroid antibodies prior to the study showed an increase in antibody titre and transient thyroiditis after administration of GM-CSF. Recently a GM-CSF/antigen fusion protein has been tested. An antibody corresponding to a specific idiotype expressed on B-cell lymphomas was fused to GM-CSF and injected into mice with B-cell lymphoma xenografts. The mice developed antibodies to the lymphoma and there was a protective effect against disease progression. Preliminary results of clinical trials using GM-CSF in humans suggest that it enhances antibody responses to hepatitis B vaccine. On the basis of these preliminary results, several clinical trials are being planned and it would appear that GM-CSF has potential as a vaccine adjuvant.

Publication Types:

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